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Review

Semisynthetic resorbable materials from hyaluronan esterification

Davide Campoccia[a], Patrick Doherty[c,*], Marco Radice[b], Paola Brun[b], Giovanni Abatangelo[b], David F. Williams[c]

[a]*Fidia Advanced Biopolymers, Via Ponte della Fabbrica 3/a, Abano Terme (PD), Italy*
[b]*Institute of Histology and Embryology, University of Padua, Deptartment of Biology, Viale Trieste, Padua, Italy*
[c]*Department of Clinical Engineering, University of Liverpool, Royal Liverpool Hospital, P.O. Box 147, Liverpool L69 3BX, UK*

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Abstract

In recent years, research on new, biocompatible, degradable materials has seen the development of a series of modified natural polymers. Among these, a new class of materials consisting of different hyaluronan derivatives promises to be useful in a whole range of clinical applications thanks to their varied biological properties. These new materials are obtained by chemical modification of purified hyaluronan consisting of the partial or total esterification of the carboxyl groups of this natural polymer. This review on the properties of the new materials reports some of their biocompatibility and characterization aspects based on findings from studies conducted on the ethyl and benzyl hyaluronan esters, two representative members of this new class of compounds, and is intended to arouse interest in the potential of other, as yet unexplored derivatives. From the results of a number of investigations, the various derivatives appear to possess different physico-chemical properties, especially as far as the degree of hydration and polymer stability are concerned. In addition, the type of esterification and extent of chemical esterification of hyaluronan considerably affects the biological properties of these materials, offering a range of polymers either favouring or, conversely, inhibiting the adhesion of certain types of cell. © 1998 Published by Elsevier Science Ltd. All rights reserved

Keywords: Hyaluronan; Hyaluronan esters; Degradable biopolymers; Resorbable materials; Hydrogels; Cell adhesion; Leukocyte activation; Tissue engineering; Drug release; Artificial cartilage; Artificial skin; Biocompatiblity; Degradable scaffolds

1. Introduction

It is obvious from the recent literature on clinical engineering [1–3] that there is an increasing interest in resorbable biomaterials with specific biological properties and good biocompatibility profiles. Much of this interest has been stimulated by recent breakthroughs in tissue-engineering techniques, where resorbable scaffold materials are used as a support matrix or as a substrate for the delivery of cultured cells or for three-dimensional tissue reconstruction [4–6]. New applications are emerging for biomaterials in addition to traditional ones where a degradable material may be used on its own, as for bone fixation in orthopaedic practice, cavity filling materials and sutures in surgery, drug delivery systems in pharmacology and so on. This means that there is a demand for degradable polymers with new physical, mechanical, chemical and biological properties. In particular, as far as the biological aspects are concerned, the issue of cytocompatibility with all its connotations is becoming of fundamental interest.

In the recent history of biopolymers, different molecular engineering strategies have led to the development of a variety of synthetic and modified natural polymers aimed at reaching the highest level of compatibility in the physiological environment, i.e. optimal performance of function, low toxicity, convenient degradation rate and ideal tissue response.

One common belief among many scientists trying to develop new biocompatible biodegradable polymers has been that these should be natural molecules or, alternatively, analogues of natural polymers, which should release degradation products that enter the normal metabolic pathways, rather than being xenobiotic. One first approach considered polymers which undergo hydrolytical degradation producing metabolites normally present in the physiological environment. Polyglycolic acid

* Corresponding author.

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(PGA) and poly-L-lactic acid (PLLA), respectively, release glycolic acid and lactic acid during in vivo degradation. Alone or combined in copolymers, they currently represent some of the most widely used degradable materials.

On the other hand, the poor physical properties of natural polymers that are soluble or rapidly degrade appear to restrict the possibilities of their exploitation in the medical field [5]. Analogs of the extracellular matrix have been produced from collagen and glycosaminoglycans which are widely and abundantly distributed in the body, but in order to achieve a certain stability of the device, it has often been necessary to modify these natural molecules chemically. The work of various groups has made it possible to obtain insoluble polymers from ECM components such as collagen by the cross-linking reaction. However, in a number of cases concern has been expressed for the potential toxicity of some of the cross-linking agents utilized, such as glutaraldehyde, formaldehyde and isocyanates [7].

An alternative solution to produce insoluble molecules starting from natural components was explored in the mid 1980s and led to the development of hyaluronan esters [8–9]. These novel hyaluronan-based materials were obtained by the esterification of the free carboxyl groups of glucuronic acid present along the chain backbone. A wide range of alcohols could be employed in many different combinations, giving rise to a variety of molecules with distinct physico-chemical and biological properties, most of which have still to be explored. This possibility would widen the choice of degradable biopolymers for medical applications offering new alternative semisynthetic molecules with easy industrial processability.

2. Hyaluronan

The large number of papers on the medical uses of hyaluronan demonstrate the significant interest generated by this natural polymer. A polysaccharide of the ECM, it plays a multi-task role, having many structural, rheological, physiological and biological functions in the body. Hyaluronan (HA), formerly known as hyaluronic acid, is a main glycosaminoglycan (GAG) ubiquitously distributed in the extracellular space, particularly in the ECM. It is a linear and monotonous anionic polymer. Two modified sugars, glucuronic acid and *N*-acetylglucosamine, form each of the disaccharide units, resulting in a chain with the following molecular structure: –[D-glucuronic acid (1-β-3) *N*-acetyl-D-glucosamine (1-β-4)]$_n$– (Fig. 1) [10].

HA preparations from tissues are polydisperse with respect to the molecular mass. Usually, the extracted HA has an average molecular weight of several million Daltons. Far from being a simple space filler, it plays different roles in the physiological environment. It is a soluble molecule forming highly viscous solutions in water. HA is heterogeneously distributed in various tissues, in some places reaching concentrations of some mg ml^{-1} [11]. Normally present in the ECM, it not only participates as a simple structural component of the three-dimensional ECM molecular network and interacts with binding proteins, proteoglycans and other active molecules such as growth factors, but also actively contributes to the regulation of the water balance acting on the osmotic pressure and flow resistance and selectively sieving the diffusion of plasma and matrix proteins [10]. In other areas, owing to its high viscosity, it behaves like a lubricant protecting the articular cartilage surfaces from shear stress. At a molecular level, HA performs an additional function, being a scavenger molecule for free radicals [12–13]. Besides all these properties, HA is recognized by specific cell receptors such as CD44, regulating the adhesion, growth, differentiation, locomotion and activation of specific cell types, modulating inflammation, angiogenesis and healing processes, and acting as a selective and protective coat around the cell membrane [14].

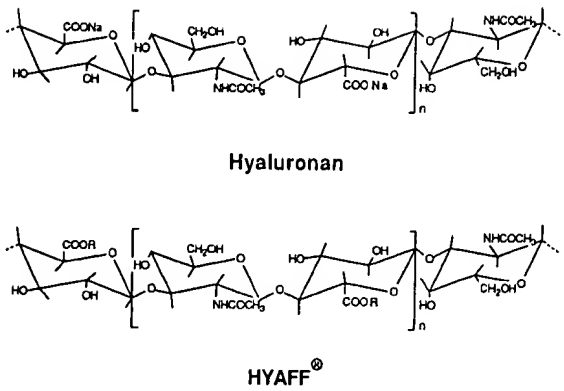

Fig. 1. Structure of HA and HYAFF® polymers. R represents one of the possible substituent ester groups.

In the last few years, the use of this fundamental polysaccharide in its highly purified form has become common practice in medicine for treatments such as the viscosupplementation of the knee and surgery to the eye. Corneal transplantation, treatment of cataract, intraocular lens implantation, treatment of vitro-retinal diseases and tympanic membrane perforation are all interesting applications in which this molecule has shown beneficial actions. Its most advantageous effects appear related to improved wound healing in the injured tissue. King et al. (1991) [15] reported that exogenous HA accelerated the healing time of holes created in the cheek pouch of hamsters. Both wound size and vascularization showed faster improvement in HA-treated animals. This may be partly explained by the known effects of HA degradation products on endothelial cell proliferation and angiogenesis [16]. All this has important implications for the

possibility of improving the healing process in wound management.

HA could certainly find additional uses, particularly in the biomaterials field, but some physical and biological characteristics of hyaluronan in its purified form, such as water solubility, rapid resorption and short residence time in the tissue, limit its possible applications. For this reason, several attempts have been made to modify its molecular structure to obtain a more stable solid material. Obviously, these were mostly oriented towards a conservative modification so as to maintain the rather inert and noninflammatory properties of high-molecular-weight, purified hyaluronan.

Cross-linking and coupling reactions were two of the ways considered to obtain a modified, stable form of HA.

New classes of insoluble polymers were developed using a variety of cross-linking agents. These chemical modifications were finalized either to trap HA chains within a net of cross-linked proteins, or to create covalent bonds between HA chains. The production of all these derivatives was driven by a concept similar to that which led to the production of cross-linked collagen [17].

For instance, the generic name of Hylan is used to indicate all those hyaluronan based materials where the cross-linking does not affect two specific functional groups of the HA chain: carboxylic and *N*-acetyl groups [18]. Hylans can be obtained by two alternative processes. One is based on the use of formaldehyde as the cross-linking agent and forms cross-links between hydroxyl groups of the HA molecule and amino or imino groups of bridge proteins. The other process, based on vinyl sulphone, creates sulphonyl-bis-ethyl cross-links between hydroxyl groups of the polysaccharide, giving rise to an infinite network of HA chains.

Along with Hylans, a variety of HA-based biomaterials have been obtained using other cross-linking agents, including: biscarbodiimides [19], carbodiimides in the presence of a nucleophile and phosphorus-containing reagents [20], poly-functional epoxides [21]. A number of photocurable cross-linked derivatives have been produced by using photoreactive reagents covalently bound to HA [22].

The list of cross-linked derivatives is even longer when one considers the materials generated either from hydrazido-functionalized [23] or partially deacetylated HA [24] and processed for subsequent cross-linking reactions. Moreover, a novel class of HA derivatives termed auto-crosslinked polysaccharides (ACP) has recently been obtained [25]. In this case too, the stability of the polymer was obtained by creating cross-linking bonds, but no additional chemicals were involved in bridging the hyaluronan chains. Stabilization was achieved by directly esterifying a certain percentage of the carboxyl groups of glucuronic acid along the polymeric chain with hydroxyl groups of the same or different hyaluronan molecules. From a toxicological point of view, the idea of having HA alone with a labile ester bond inter- or intra-chain would exclude the leaching of any toxic cross-linking agents which may be used to bridge polymer chains.

The vast majority of the described cross-linked materials are water-insoluble gels with better viscosity and chemical stability than HA, and are generally susceptible to extensive hydration in aqueous solutions.

The alternative way to obtain HA derivatives involves the use of coupling reactions where specific functional groups of the polysaccharide (e.g. carboxyl, hydroxyl, *N*-acetyl groups) are modified by chemical reactions such as esterification, sulphatation, amidation and so on [26]. All these modifications of the functional groups may have a profound effect on the original HA molecule, significantly affecting its physico-chemical and biological properties.

For instance, water-insoluble *N*-acylurea derivatives of HA forming hydrated gels have been obtained through the reaction of the carboxyl groups with carbodiimide in the absence [19] or in the presence of a polyanionic polysaccharide and a nucleophile [27].

In this review, attention will be focused on HYAFF®, another class of hyaluronan derivative polymers obtained by a coupling reaction. The strategy behind the creation of HYAFF® was to improve the stability of the polymer by esterifying the free carboxyl group of glucuronic acid, frequently repeated along the HA chain, with different types of alcohols. This chemical modification acts both by reducing the hydrophilic, negatively charged, carboxyl groups of the polyanionic polysaccharide and increasing its hydrophobic components. A broad variety of polymers can be subsequently generated either by changing the type of ester group introduced or the extent of the esterification (percentage of carboxyl groups esterified).

3. Hyaff®: polymers ranging from soluble materials to solid hydrogels

The current source of highly purified hyaluronan used for the preparation of the ester derivatives is rooster combs. The synthesis of HYAFF® from HA involves a two-step procedure: the preparation of a quaternary salt of HA and its subsequent reaction with an esterifying agent in aprotic solvent at controlled temperature. A variety of alcohols can be used for the esterification (aliphatic, aryliphatic, cycloaliphatic and others). Fig. 1 shows the general structure of HYAFF® compared to that of HA.

Once esterification of the polymer has been obtained, the material can easily be processed to produce membranes, fibres, sponges, microspheres and other devices, by extrusion, lyophilization or spray drying. The ethyl and the benzyl esters of hyaluronan, respectively termed

HYAFF® 7 and HYAFF® 11, are two of the most characterized HYAFF® polymers, from both the physico-chemical and biological viewpoints. They are produced starting from HA of about 200,000 Da and are useful in understanding the effects that changing two variables, the type of ester and the percentage of esterification, have on molecular properties. The extent of molecular modification modulates the soluble and viscous nature of purified hyaluronan in aqueous solution and has proved to have profound effects on the interaction of hyaluronan with water. It was found that the higher the percentage of esterification of hyaluronan, the lower its solubility in water [28]. This was observed with ethyl, propyl, benzyl and other alcohols, but it is thought to be generally true for many other alcohols presenting a hydrophobic chain. By masking the free carboxylic group, the double goal of both reducing the anionic charge of hyaluronan and increasing the hydrophobic parts along the hyaluronan chain may be achieved. The results of this modifications are, along with reduced solubility as may be expected, a slight increase in structural rigidity caused by the interaction of hydrophobic groups, becoming organized in hydrophobic patches, which affects the flexibility of the chain. Despite these changes, X-ray diffraction studies on benzyl alcohol derivatives indicate a diffraction pattern typical of unsubstituted HA.

Examining in detail the properties of benzyl and ethyl HA, the derivatives where 100% of the carboxyl groups are esterified (benzyl and ethyl total esters), it emerges that they are insoluble transparent materials which can be extruded into thin membranes. This form of material was utilized for most of the physico-chemical characterization studies.

A series of ESCA and infrared analyses was carried out on derivatives in the form of membranes differing in their percentage of esterification and type of esters [28]. The results indicate that the surface composition of all the samples differs from that of the bulk, the hydrophilic–hydrophobic character of the samples changing according to the chemical composition as shown by ESCA and contact angle measurements. Both infrared and contact angle measurements revealed that surface restructuring occurs upon hydration and the greater the hydrophilic character of the sample, the greater and faster the restructuring phenomenon. Examining the materials' behaviour in aqueous solution, the total benzyl ester (HYAFF® 11) showed only slight hydration when placed in buffered phosphate saline solution (about 40% increase in weight), while the total ethyl ester under the same conditions (HYAFF® 7) underwent more extensive hydration (about 235%). Additional investigations on these and other total esters were conducted considering polymer hydration in water at 37°C for 1 h. The degrees of hydration observed for ethyl, *n*-propyl, *n*-pentyl, benzyl and dodecyl esters were respectively: 259, 168, 43, 48, and 19%. In the case of 75% hyaluronan benzyl and ethyl esters (respectively HYAFFA® 11p75 and HYAFF® 7p75) hydration was even greater, reaching up to 1250% of weight increase with respect to the dry conditions. On approaching 25% of esterification, the esters become largely soluble in water. The relation between percentage of esterification/level of polymer interaction with water associated with the possibility of controlling stoichiometrically the degree of esterification offers the possibility of producing derivatives with defined properties.

The transport properties of films formed from hyaluronic acid esters were investigated in a further study, where the permeability of the films to various solutes, the rate and extent of hydration, and the release of a model compound were used as indicators of transport behaviour. Membranes of ethyl and benzyl total esters, respectively HYAFF® 7 and HYAFF® 11, demonstrated permeability to neutral solutes of over 1×10^{-5} cm s^{-1}. Chlorpromazine was used as a model compound of release following physical incorporation into the films. Complete release of the substance took less than 200 min [29]. A correlation between permeability and degree of polymer hydration was observed, suggesting that the solute may be transported through these hydrated films by diffusion in aqueous channels or pores. In hydrated membranes of total and partial benzyl esters, Joshi and Topp [30] found good correlation between the percentage of freezable water content determined by differential scanning calorimetry and permeability coefficients for various model compounds. This finding suggests that the model solutes may be transported primarily in freezable water.

As far as the thermomechanical properties of hyaluronan esters are concerned, a series of studies were conducted by Iannace et al. [31] using fibre samples of different derivatives. In these studies the thermal properties of the total ethyl and benzyl esters were found to be connected with the polysaccharide structure of the molecules, and poorly influenced by the substituents. The thermograms obtained by differential scanning calorimetry for both HYAFF® 7 and HYAFF® 11 evidenced three different peaks: the first one related to a loss of water from the material and the other two connected to a complex phenomenon of thermal modification, finally leading to the formation of carbon. The curves from the thermogravimetric analysis confirmed a loss of weight related to changes in water content of the materials from 25 to 100°C, thermal decomposition with intensive loss of mass from 225 to 280°C and, thereafter, a deceleration of the loss of mass.

The analysis of static and dynamic mechanical properties of HYAFF® and HYAFF® 11 indicated that the two polymers possessed good mechanical strength in the dry state. A significant decrease in strength was observed for the wet materials. The fundamental role of the substituent on the microstructure is believed to be the modulation of the hydrophobic and hydrophilic microdomains

responsible for the interactions with water molecules, consequently inducing variations in the mechanical properties [31].

4. Semisynthetic polymers with easy processability

An important advantage of HYAFF®-based polymers is their easy processability. Indeed, once modified, the hyaluronic acid molecule can be extruded to produce fibers or thin transparent membranes, lyophilized to obtain sponges, or treated by drying, extraction and evaporation to produce microspheres. Furthermore, HYAFF® fibers can be worked into gauzes, ropes or non-woven structured materials. Other more recent techniques allow the coating of medical device surfaces following activation by plasma treatment.

Depending on the starting derivative, the object will retain different biological properties and susceptibility to resorption. Certainly, much remains to be explored about the possible developments of this broad class of polymers.

5. In vitro degradation profile of HYAFF®

The mechanism by which resorbable materials are degraded is very important. As explained above, it is fundamental that no toxic species be generated. In order to define the stability and degradation mechanisms of HYAFF® materials, a series of in vitro studies were performed at F.A.B. srl Research Laboratories. Samples of thin films of total and partial esters were incubated in artificial plasma at a concentration of approximately 1 mg ml^{-1} at 37°C until complete degradation occurred. The presence of benzyl alcohol in the incubation medium and in hydrolysed incubation medium was analysed by HPLC using a C18 column and a UV detector, while the presence of hyaluronan was estimated by quantifying the *N*-acetyl-glucosamine sugar. The results concerning the benzyl esters HYAFF® 11 and HYAFF® 11p75 (Figs. 2 and 3) show that under the test conditions both materials underwent hydrolytic degradation of the ester bonds even in the absence of any enzymatic activity. The spontaneous hydrolysis of the ester bonds was mostly completed after 1–2 weeks in the case of the hydrated HYAFF® 11p75, while it required on the order of 2 months for the more stable HYAFF® 11. Findings similar to that observed for HYAFF® 11, indicating slow degradation over a period of about 2–3 months, were encountered examining the release of ethyl alcohol from the total ethyl ester HYAFF® 7.

These results suggest that the hyaluronan esters undergo spontaneous deesterification when in an aqueous environment, while from the quantification of the presence of *N*-acetyl-glucosamine the hyaluronan backbone chain

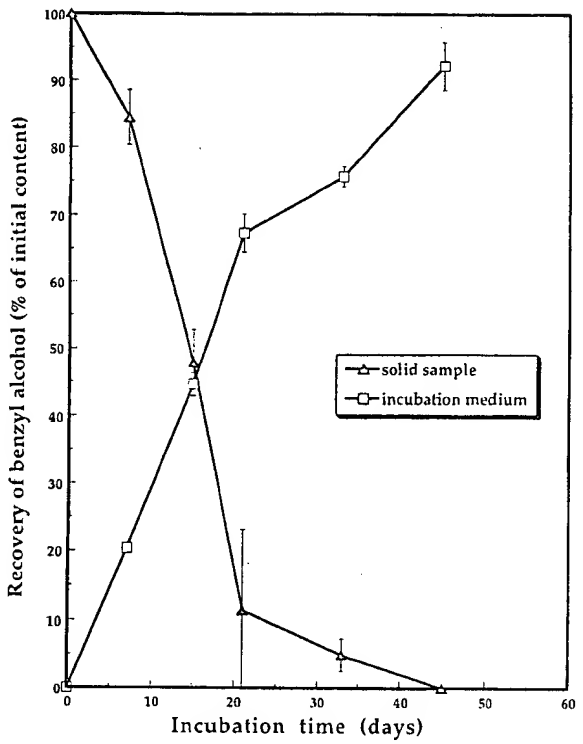

Fig. 2. In vitro degradation of HYAFF® 11 films: recovery of benzyl alcohol in solid material and incubation medium.

appears to be more stable under the same conditions. It has to be underlined that such a mechanism of release is expected to result in a progressive loss of esterification of the polymer which subsequently becomes more hydrated and soluble, and increasingly similar to HA. The degradation rate is significantly influenced by the degree of esterification probably because partial esters are more flexible and hydrated than completed esterified esters in which the hydrophobic patches make the polymer chain network more rigid and stable. Further evidence of this degradation mechanism was collected from experiments, described in more detail further on, investigating the kinetics of drug delivery using substances covalently bound to HA.

6. Effects of derivatization on the biological properties exhibited by hyaluronic-acid-based materials

The first observations on the biological effects of HYAFF® were made using powders and thin membranes of material placed in contact with different cell types under in vitro conditions. In 1991, Cortivo et al. [32] demonstrated that when culturing murine fibroblasts in the presence of ethyl and benzyl derivatives, the cells were not affected or altered by the treatment. Subsequently, other qualitative and quantitative cytotoxicity studies, where the materials were tested by direct contact

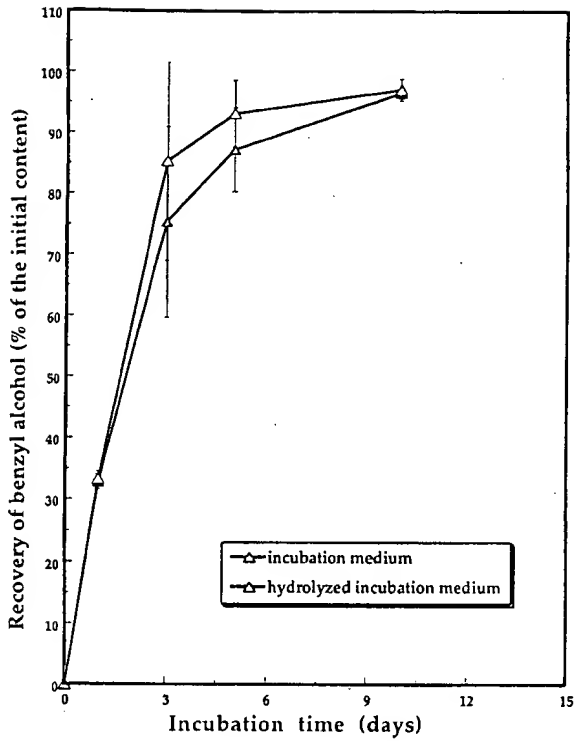

Fig. 3. In vitro degradation of HYAFF® 11p75 films: recovery of benzyl alcohol in incubation medium and hydrolyzed incubation medium.

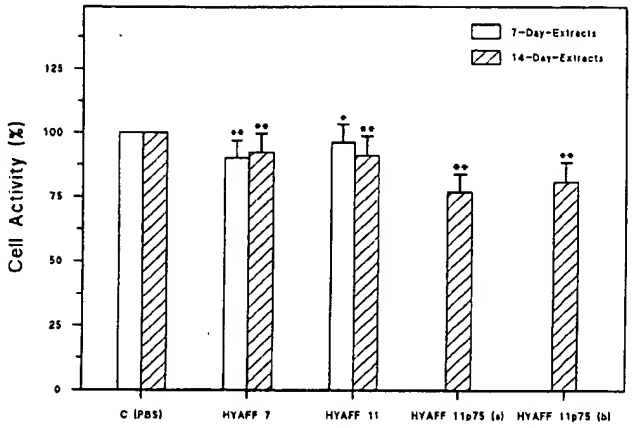

Fig. 4. Effects of extracts of films of HYAFF® 11, HYAFF® 7 and HYAFF® 11p75 in phoshate buffered saline solution (ratio material surface/volume = 4 $cm^2 ml^{-1}$; incubation temperature = 37°C) on L929 fibroblast-like cells. The results of two different lots of HYAFF®11p75 films, 14/90 (a) and 9/91 (b), are reported. (One way-ANOVA: * = $P < 0.001$; ** = $P < 0.001$).

test or their extracts were assayed by an MTT-based quantitative technique derived, with minor adaptations, from the original method developed by Mossman [33], indicated little or no effect on cell viability (Fig. 4). Generally, the more stable total esters were found to be non-cytotoxic, while the partial esters such as HYAFF® 11p75, which undergo rapid degradation during the extraction period, were found to cause minor inhibition in the cellular metabolism measured as succinate dehydrogenase activity.

After assessing cell viability, attention was focused on cell behaviour, trying to understand how cells interact with the smooth surfaces of the different polymers. It was found that both the variables (type of ester and percentage of esterification) were involved in determining the level of cell adhesiveness of the surface.

It is already known that HA promotes adhesion in mixture with collagen but only at concentration of below 5%, over which its effect becomes the opposite, inducing the cells to become round [34]. On the basis of such observations conducted using fibroblast cells, polymers with little esterification may be expected to behave like a substrate with locally very high concentrations of HA.

Derivatives with as little as 25% of esterification were generally found to be soluble, but as the esterification increases to values around 50%, as in the case of the 50% benzyl ester of HA (HYAFF® 11p50), the materials become more stable and interact with water to a lesser extent. At this point we have a solid material resembling a hydrogel. Experiments using fibroblastoid L929 cells showed that the material is recognised as a spot of highly concentrated hyaluronan and most cells on the material surface are unable to adhere or spread. The 75% benzyl ester too (HYAFF 11p75) exercises a similar effect on the cells, allowing them to attach only minimally (Fig. 5a and 6). Only over a threshold of about 80% of esterification is polymer hydration reduced. The material becomes more stable and allows the cells to attach, but the optimal degree of cell adhesion and spreading is reached only in proximity to total esterification. This is the case of the total benzyl ester HYAFF® 11 (Fig. 5b). Fibroblastoid cells adhering on HYAFF® 11 exhibit regular morphology but their shape is less flattened when compared to cells on the surfaces of tissue-culture-grade polystyrene plastic controls (Fig. 7a and b).

The kinetics of L929 cell adhesion onto HYAFF® 11 surfaces were very similar to those of the control, reaching a plateau after about 2 h of incubation, although the number of cells on the HYAFF® 11 at this time was slightly less than that on the control (Fig. 6)

Different behaviour was observed with the ethyl ester. In this case, when reaching total esterification as in HYAFF® 7, the material remains highly hydrated as described previously, and the cells, even when attached, show round morphology and sometimes aggregate in clusters, preferring their own surface to that of the polymer (Fig. 5c). Cytotoxicity studies indicate that the inability of the cells to attach to the surface is not associated with death or any impairment of cellular functions.

These and other facts would suggest a possible link between high degree of hydration—low cell adhesion, as

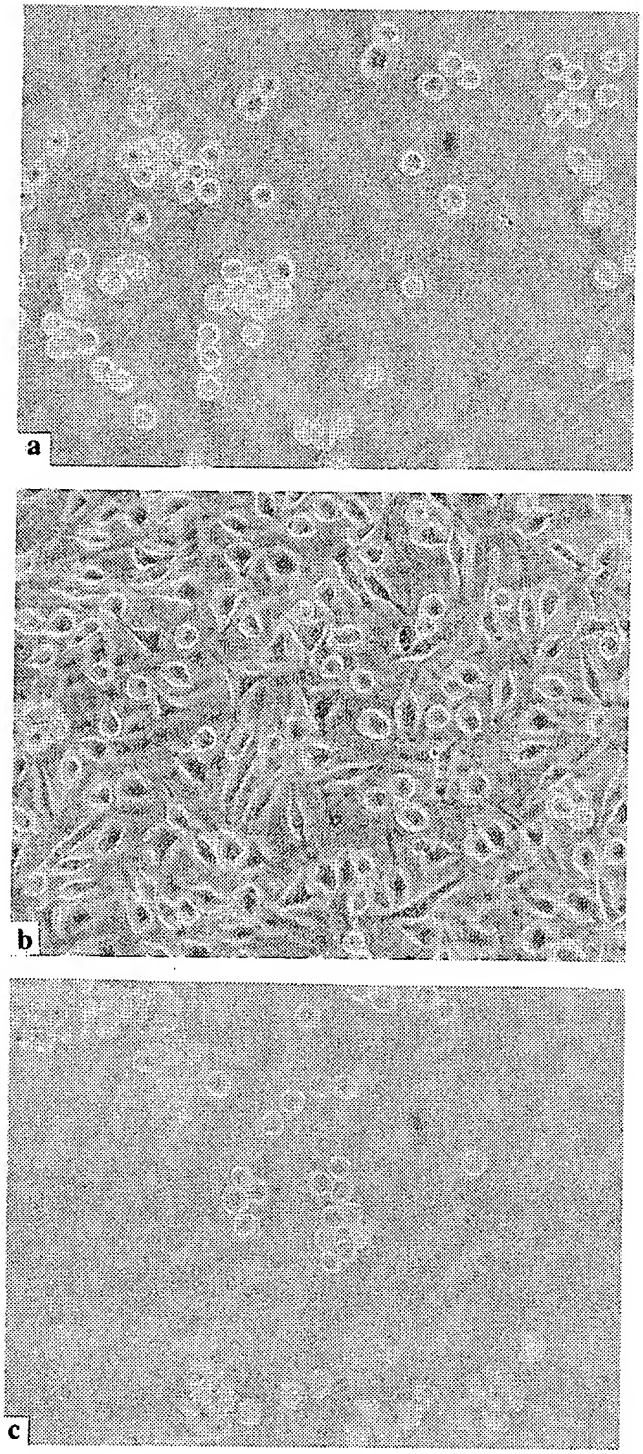

Fig. 5. Phase-contrast micrographs of L929 cells seeded onto film surfaces of HYAFF® 11p75 (a), HYAFF® 11 (b) and HYAFF® 7 (c).

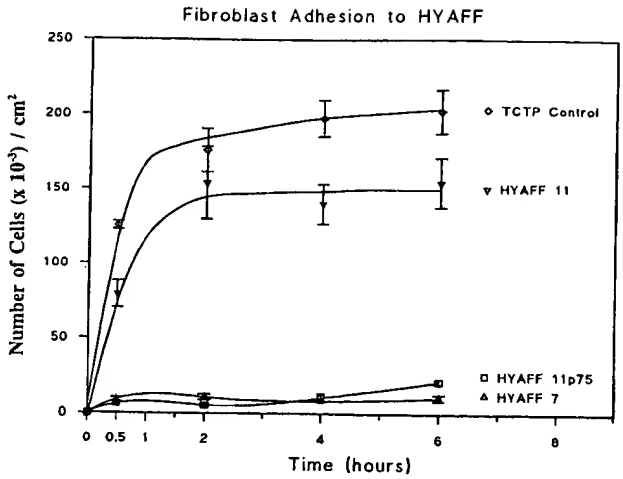

Fig. 6. Kinetics of L929 cell adhesion onto different HYAFF® surfaces and tissue culture treated polystyrene (TCTP).

has previously been suggested by Lydon et al. [35] following experiments on the properties of different synthetic materials. A surface mainly composed of water as in the case of HYAFF® 11p75, which reaches over 1250% hydration in weight, may offer only a reduced number of surface points where proteins can adsorb and cells are able to adhere via receptors. However, we do not want to exclude the possibility that, in the presence of specific functional groups or cationic charges (e.g. chitosan), cell adhesion may not be impeded even on strongly hydrated substrates.

Other factors which might be assumed to have an influence on cell adhesion are the surface microstructure and energy. However, examinations under scanning electron microscopy showed that all the HYAFF® membranes have rather smooth surfaces with only minimal grooving due to the manufacturing process, and are believed not to influence cell adhesion. Barbucci et al. [28] reported some measurements of contact angles obtained both by placing a drop of fluid on a plate of the material and by the inverted air bubble within a fluid phase, in this case the latter being water. They found a difference between the lower and the upper face of the hydrated film specimens of the same material. The measurements of contact angles on HYAFF® 11 and HYAFF® 7 were found to be similar when the most hydrophobic and hydrophilic sides were compared.

However, when contact angles were measured under static conditions in a more physiological buffered saline phosphate solution no difference was found between 7 and HYAFF® 11 and both materials evidenced a highly hydrophilic nature following complete hydration of the samples (for HYAFF® 7 and HYAFF® the contact angles by means of the captive air bubble were found to be 12°C and 13°, respectively). Therefore, the difference in fibroblast adhesion does not seem to be associated with differences in wettability or structure.

The situation changed when the studies were conducted with elicited primary rat macrophages in place of fibroblasts. In this case too, the more hydrated total ethyl ester HYAFF® 7 allowed the cells to adhere and spread

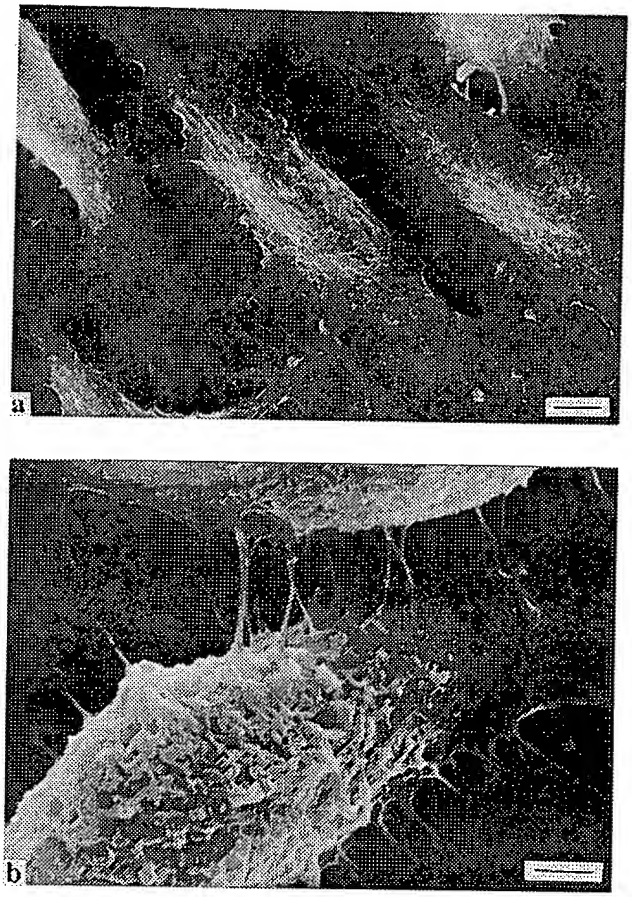

Fig. 7. SEM micrographs of L929 cells adhering onto material surfaces of tissue culture treated polystyrene ((a) bar = 2 μm) and HYAFF® 11 ((b) bar = 1 μm).

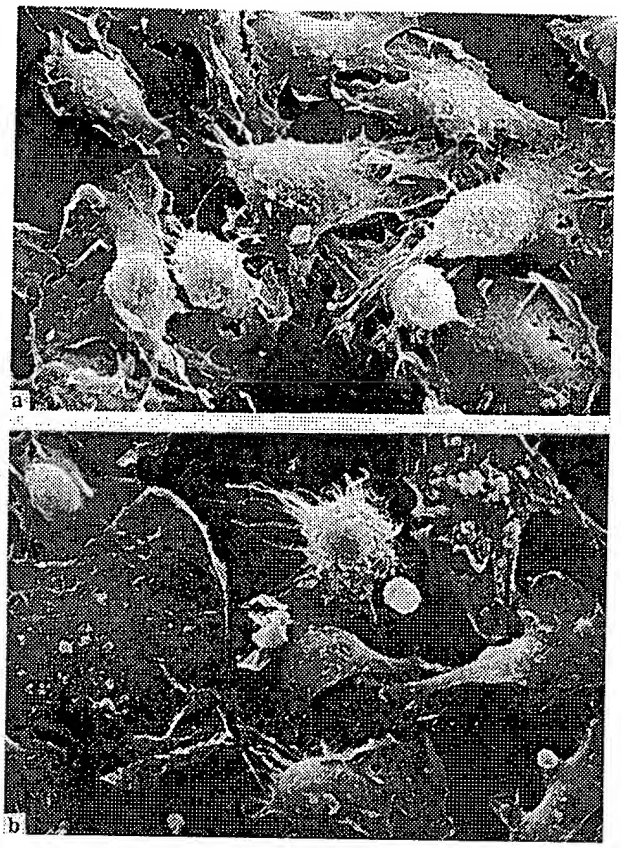

Fig. 8. SEM micrographs of rat peritoneal cells adhering to HYAFF® 11 (a) and HYAFF® 7 (b).

extensively, and no obvious difference was observed with respect to the same cells on the total benzyl ester HYAFF® 11 (Figs. 8 and 9).

Even more curious was the behaviour of human neutrophils, which showed a round morphology on the inert HYAFF® 11, while they became polarized and motile when placed on the surface of HYAFF® 7, clearly indicating a certain potential of the latter in inducing neutrophil chemokinesis [36].

The observations from these different studies on material–cell interactions support the idea that it is incorrect to describe a material as adhesive without mentioning or referring to the specific cell type used in the investigations. Furthermore, changing the type of chemical modification may make it possible to develop material surfaces with specific activity with respect to cell adhesion and metabolic activation.

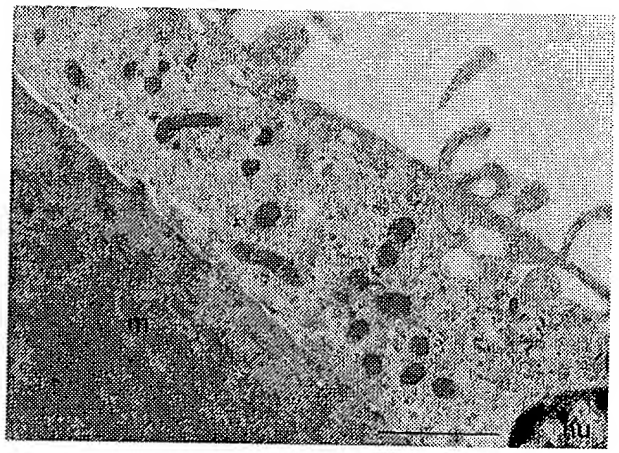

Fig. 9. TEM micrograph of a rat peritoneal cell, probably a macrophage, on the surface of a film of HYAFF® 11 (bar = 1 μm, *m* = material).

7. Leukocyte interactions with HYAFF®

It has been mentioned above that membranes of benzyl and ethyl esters induce different behaviour in human neutrophils seeded onto their surfaces. Additional experiments considered the effects on these cells by different soluble polymers. In particular, studies were carried out to determine the capability of hyaluronan derivatives to

cause neutrophil polarization, i.e. to induce cells to change from an initial round shape, normally observed in resting neutrophils, to an elongated morphology, often indicating chemokinetic activity [36]. Many substances, among them some metals, some endotoxins, bacterial peptides, complement components and other chemotactic factors, are known to alter neutrophil morphology [37]. Increased cell locomotion and chemotaxis often result in a local inflammatory response with leukocyte accumulation. The next step was obviously to determine what kind of direct effect soluble molecules released from the biomaterial bulk would have. In Table 1 some results concerning this type of study are reported. It is possible to note that while the majority of the control cells, suspended only in buffered saline solution, exhibit a round morphology and less than 5% of them show polarization, most of the positive control cells stimulated with formyl-met-leu-phe (fmlp), an active chemoattractant peptide, became polarized. Our data indicate that, in the range of molecular weights and concentrations tested, HA appears to be inactive in causing the cells to polarize. Polymer suspensions of partial derivatives such as the 75% benzyl ester HYAFF® 11p75 were found not to alter the cell morphology. These results suggest the absence of a direct chemotactic effect of molecules leaching from the bulk of HYAFF® 11 and HYAFF® 11p75-based biomaterials.

The activation of macrophages represents another informative aspect of the biomaterial–leukocyte interaction, particularly in the case of degrading material which normally releases significant amounts of leachable molecules. J774 murine cells having a macrophage-like phenotype were chosen to assay the cell activation potential of HYAFF® 11, membranes and non-woven structured materials of HYAFF® 11p75 and HYAFF® 7. Tumour necrosis factor (TNF) and nitrogen monoxide were chosen as markers for metabolic cell activation. The use of J774 cells was preferred to that of other secondary human cell lines which normally present a less sensitive response and require priming with phorbolmyristic acid (PMA) to produce TNF and nitrogen monoxide following induction with bacterial lipopolysaccharide.

The standard tests for both markers were modified to be conducted in 96-well plates at a cell density of 10^6 cells ml^{-1} (200 µl $well^{-1}$). The exposure to the test polymers was carried out seeding the cells on dishes of about 5 mm in diameter, obtained by biopsy punch from HYAFF® membranes, and placed on the bottom of the wells. The cells were exposed to the environment for 4 h and 24 h, respectively, for the TNF and nitrogen monoxide measurements. For the assessment of TNF, a bioassay on L929 cells sensibilized by actinomycin-D and LiCl was used [38], while the nitrogen monoxide was determined using the reagent of Griess after the conversion of nitrogen monoxide to nitrites [39]. In both cases an internal calibration curve was prepared.

The tests were independently conducted for the two markers. The results summarised in Figs. 10 and 11 suggest that HYAFF® 11 does not cause any significant increases in the production of the selected activation markers by J774 macrophages. HYAFF® 11p75 had only a slight effect, while HYAFF® 7 induced a significant rise in the production of both TNF and NO (6.9 ± 2.0 µM).

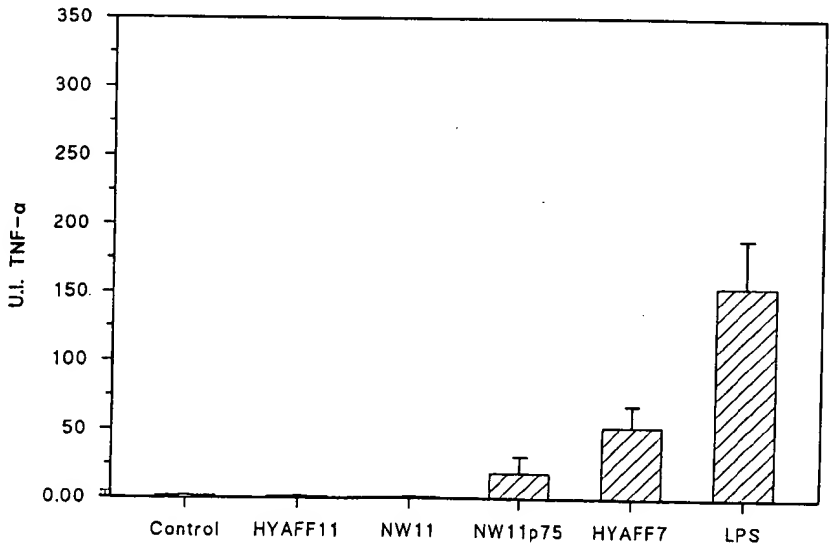

Fig. 10. Production of TNF by J774 cells seeded onto different HA derivatives: film surfaces of HYAFF® 11 (HYAFF11) and HYAFF® 7 (HYAFF® 7), and non-woven structured materials of HYAFF® 11 (NW11) and HYAFF® 11p75 (NW11p75). The negative and the positive controls were obtained incubating the cells respectively with only medium (control) or with medium supplemented with 1 µg ml^{-1} of lipopolysaccharide from *Salmonella minnesotae* (LPS).

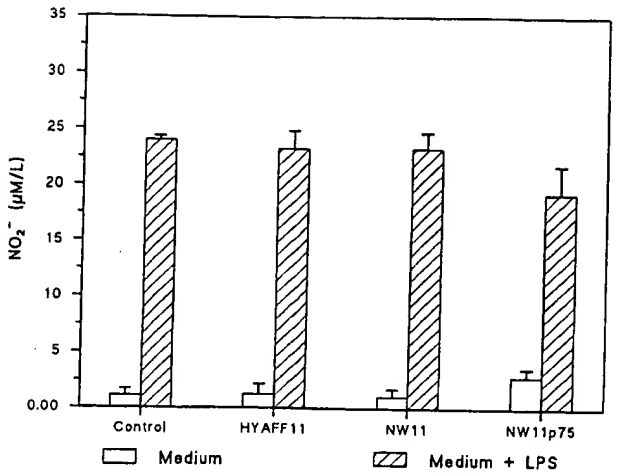

Fig. 11. Production of NO by J774 cells seeded onto different HA derivatives: films of HYAFF® 11 (HYAFF® 11), non-woven structured materials of HYAFF® 11 (NW11) and HYAFF® 11p75 (NW11p75). The experiment was performed in the presence and in the absence of 1 μg ml^{-1} of lipopolysaccharide (LPS). The reduction in NO production when the cells were seeded onto NW 11p75 and stimulated with LPS may be due to a slight metabolic inhibition which has been observed also on L929 cells in the MTT-based cytotoxicity test.

8. Effects of hyaluronan derivatives on complement activation and fibrinolysis

The interactions between biomaterials and cells may involve the direct interaction of cell receptors with specific molecules exposed on the surface of the material and/or a series of indirect interactions which are mediated by phenomena such as protein adsorption or alterations in the equilibrium of major events in the humoural tissue response, such as the coagulation and complement cascades. The complement system is an effective mechanism of the immune system which accomplishes many different tasks, among them: recognition of infective agents and foreign surfaces in the tissues, targeting of an object to enhance leukocyte migration and activation processes (e.g. phagocytosis and degranulation) and, finally, induction of cytolysis. The complement activation process includes an enzymatic cascade in which about 20 different proteins are involved. Even though activation of the complement cascade is usually considered relevant only to the characterization of the haemocompatibility of materials applied in direct contact with blood, it was thought important to understand the way these new polymers derived from HA interact with the complement system. Therefore, a detailed study was conducted to investigate the effects of hyaluronan derivatives at different levels of the complement cascade. To reach this objective, the production of C4d, Bb, iC3b and SC5b-9 complement components was evaluated. C4b was the marker for the activation of the classical pathway, Bb for the alternative pathway, and iC3b and

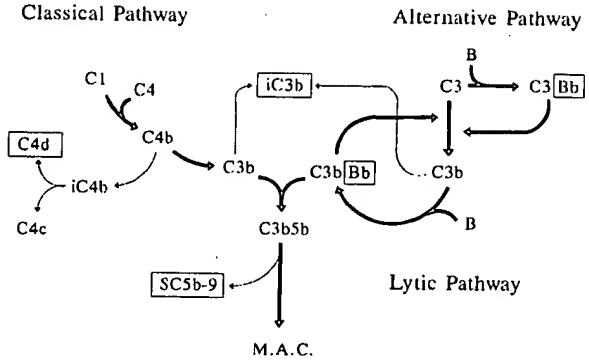

Fig. 12. Scheme of the complement activation cascade up to the formation of the membrane attack complex (MAC) showing the points of production of the selected the complement activation markers used in this study.

SC5b-9 for the final lytic pathway (Fig. 12). All the selected components were quantified using FDA approved enzyme immunoassay kits purchased from Quidel. Plasma was isolated from 20 ml of fresh heparinised blood from healthy volunteers by centrifuging at 400 g at 4°C for 15 min. It was stored on ice and used in the first 2 h after isolation. The total volume of plasma used for the reaction was 0.5 ml. Sterile eppendorfs were prepared placing 8 square specimens of 5 × 5 mm (only 4 specimens per eppendorf were used to test the production of C4d fragment). The samples were incubated for 1 h at 37°C under constant agitation in a circular mixer, supplemented with an equal volume of stabilizing solution (Quidel), immediately frozen and stored at −80°C.

The fact that polyanions, such as nucleic acids, heparin, dextran sulphate and chondroitin sulphate, have in the past been reported to cause activation of the classical pathway acting on the cationic C1q subcomponent of C1 [40, 41] might lead us to think the HA partial esters with a high percentage of free, negatively charged carboxyl groups as potential activating materials. However, it must be said that, despite these known effects of polyions at the level of the classical pathway, heparin and other polysulphonated polymers have also been found to have anticomplementary properties at an alternative pathway level. For instance, surface-bound heparin has been shown to inhibit complement activation by increasing the ability of factor H to bind C3b on the heparin-coated surfaces, while heparin in the fluid phase has been found to interfere with the binding of factor B to C3b [42].

Our results show only minimal variations in the plasma concentration of C4d following the treatments with HYAFF® 11, HYAFF® 11p75 and HYAFF® 7 (Fig. 13). The values found never exceed the normal physiological range of 0.7–6.3 μg ml^{-1} (3.5 μg ml^{-1} ± 2SD) (as reported in the technical sheet for the EIA kit by Quidel, following the measurement of the fragment in 80 normal EDTA plasma specimens); consequently, such values should not be considered significant alterations.

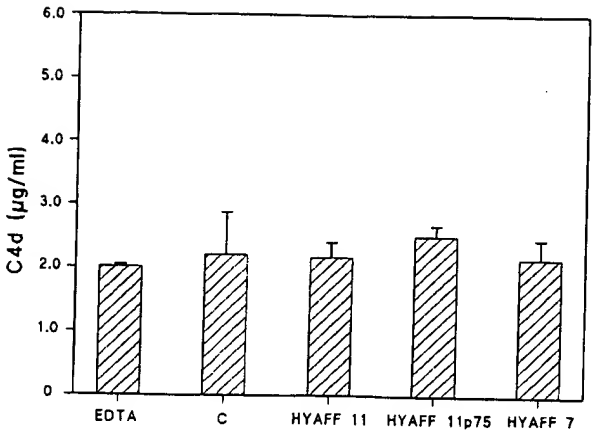

Fig. 13. The production of C4d by HYAFF® 11, HYAFF® 11p75 and HYAFF® 7, compared to untreated (C) and EDTA-treated plasma (EDTA).

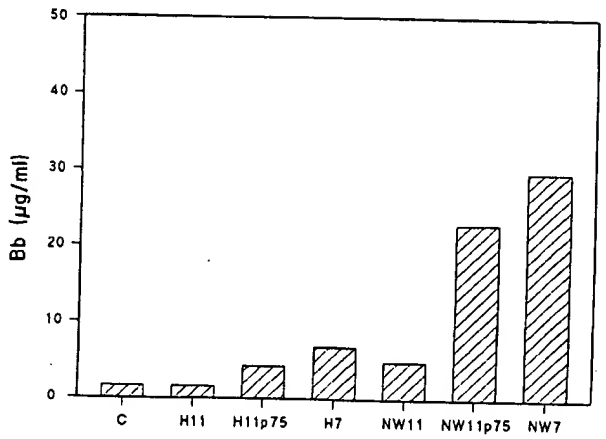

Fig. 14. The production of Bb complement component by films of HYAFF® 11, HYAFF® 11p75 and HYAFF® 7, and non-woven structured materials of HYAFF® 11 (NW11), of HYAFF® 11p75 (NW11p75) and of HYAFF® 7 (NW7), compared to untreated plasma (C).

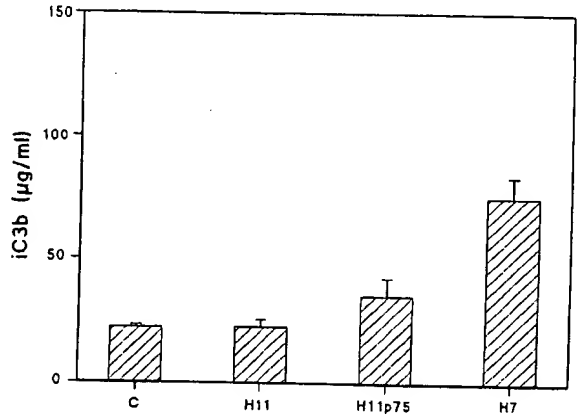

Fig. 15. The production of iC3b complement component by films of HYAFF® 11, HYAFF® 11p75 and HYAFF® 7, compared to untreated plasma (C).

Under the test conditions used, no HA derivative appeared capable of significantly triggering the classical pathway when suspensions of partial benzyl and ethyl esters were used in place of film specimens. From such results it seems that the anionic charges on these polymers are not effective in initiating the classical pathway cascade. This fact may be due to the lower negative charge of these molecules with respect to DNA, heparin and the other above-mentioned known activators, which possess phosphate or sulphate groups. It may also be that partial derivatives have only a very weak activity and that higher concentrations are required in order to observe noticeable activation.

The concentration measurements of Bb and iC3b fragments, and SC5b-9 complex showed similar results (Figs. 14–16). Both HYAFF® 11p75 and HYAFF® 7 were found to be capable of causing complement activation up to the final production of the lytic complex. Films of HYAFF® 11 have an inert surface and do not interact with plasma altering the equilibrium of the complement cascade. With respect to the control no variation or only slight differences were noticed in assaying the benzyl total ester for Bb, iC3b and SC5b-9. Upon examination of SC5b-9, films of HYAFF® 11p75 caused significant effects on complement activation similar to those of HYAFF® 7.

The hyaluronan derivatives used in this study appear to be incapable of triggering the classical pathway. The exposure of plasma to partial esters and the ethyl total ester resulted in the activation of the alternative and lytic pathways. It was, therefore, thought that these materials might act prevalently at the alternative pathway level, as confirmed by the studies considering the production of the Bb fragment to verify the activation of this part of the cascade. Interestingly, complete esterification of HA with benzyl alcohol suppresses any expected reactivity. Cellulose-derived polymers and other carbohydrate derivatives (e.g. sepharose and sephadex) are known activators of the alternative pathway [43]. It is possible to hypothesize that hydroxyl groups, which are numerous in polysaccharide molecules, play an important role in altering the equilibrium of the alternative pathway. Indeed, these groups can interact with the thioester active site of C3b which, consequently, becomes covalently bound and available to form the enzyme C3 convertase. Once C3b is anchored to the material surface, the activation proceeds via the formation of $C3b(H_2O)Bb$ and C3bBb convertases. The binding of the B component to the anchored C3b is believed to be a crucial point in material-induced activation. The competitive inhibitory mechanism regulated by factor H has an analogy function.

Only materials which bind C3b, favouring its interaction with B, will cause a significant activation, while materials that allow and promote the interaction of C3b with factor H will be less reactive, in spite of the significant binding of C3b onto the surface.

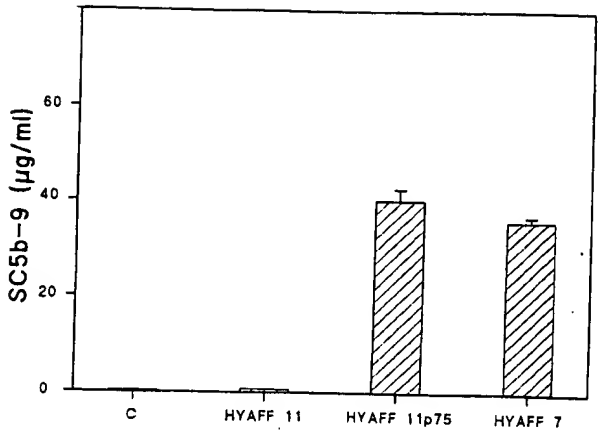

Fig. 16. The production of C5b-9 complement component by films of HYAFF® 11, HYAFF® 11p75 and HYAFF® 7, compared to untreated plasma (C).

At present, it is not clear why the totally esterified benzyl ester did not cause any, or caused only slight, complement activation, while all the partial esters and the total ethyl ester-induced significant effects. Probably, the conformational structure of the molecule and the availability of hydroxyl groups in the outer space may determine a difference in the polymer reactivity.

It should be noted that HYAFF® 11 does have numerous potentially reactive hydroxyl groups just like HYAFF® 7 and many of the partial esters. Although there are several possible explanations for this, the different capability of HYAFF® 11 influence the complement activation process may be caused by the different availability of these hydroxyl groups on the surface, or possibly by their greater binding to factor H.

From all these results, it appears that films of HYAFF® 11 do not produce opsonins or anaphylatoxins when implanted in the tissue and in contact with blood. Therefore, in the absence of these inflammatory factors the tissue response evoked by HYAFF® HYAFF® 11 is expected to be milder than that of other derivatives such as HYAFF® 11p75 and HYAFF® 7.

Usually, only biomaterials which are to come into contact with blood are tested for their effects on the coagulation cascade and complement activation. In the specific case of hyaluronan derivatives, interest in these aspects concerning blood compatibility was aroused by the studies by Le Boeuf et al. [44], which demonstrated the existence of specific binding between hyaluronan and human fibrinogen. Consequently, Scully et al. [45] decided to examine in greater detail the possibility of interaction by purified HA and some selected derivatives with the blood coagulation cascade and more specifically with fibrinolytic activity. The results indicated that HA (MW of about 200 kDa) corresponding to 2.0 mg ml^{-1} enhanced the rate of fibrin polymerization approximately twofold and the solution turbidity 3.7-fold. The enhancement was found to be concentration-dependent. The esterified derivatives were less capable of interacting with the fibrinolysis with respect to purified HA, and the greater the percentage of esterification the lesser was their activity according to the following series HA > HYAFF® 11p25 > HYAFF® 11p50 > HYAFF® 11p75 > HYAFF® 11. From these results the free carboxyl group of HA is important in determining inhibition of fibrinolysis and slight procoagulant activity. The authors suggest that such HA activities may be reduced and modulated masking the carboxyl with ester groups. Once again, complete esterification with benzyl ester groups appears to diminish the extent of material interaction with the physiological fluids.

9. In vivo tissue response to HYAFF®

A fundamental aspect of degradable materials is the type of tissue response evoked following implantation. There are at least 6 fundamental variables capable of having a profound influence on the reaction of the tissue to such materials: the site of implantation and animal species, the intrinsic reactivity depending on the material chemistry, the mechanism of chemical degradation and physical deterioration (dissolution/fragmentation), the rate of degradation and diffusibility and reactivity of the leachable degradation products, and the shape and size of the implant.

Apart from the variability due to the external factors of the animal species and the site of implantation, much of the story of resorbable materials can be determined by the type and the rate of degradation, sometimes beyond the fine chemistry of the material itself. These considerations are consistent with the experiences using ECM analogs composed of collagen and other common GAGs. The simple accumulation of polymeric material in a body site, even just normal ECM, gives rise to a local response mediated by scavenger leukocytes, normally actively involved in the catabolism and turnover of physiological components. Injected HA, which in certain body sites is subject to such rapid hydrolysis, diffusion and turnover as to compete with the prompt cellular response, may represent one of the exceptions. A week following administration in subcutaneous or intramuscular sites no or little evidence remains of the treatment.

A series of observations on the most widely used degradable polymers have more often evidenced the presence of a second delayed tissue response in addition to the initial reaction following surgery for the implantation procedure. Such a response is normally associated with the beginning of chemical and mechanical degradation of the material and involves the presence of phagocytic cells associated with the material resorption process.

There may be a long delay before the material begins to resorb, sometimes this degradation-associated

secondary response is evoked years after insertion of the implant. This has been reported in the case of slowly resorbable polymers such as polylactic acid (PLLA) [46], where the response appeared to be induced mainly by particles generated by the biodeterioration of the implant. A similar macrophage-mediated secondary response is also known to occur in the case of poly(ethylene oxide)-poly(butylene terephthalate) (Polyactive™) and other commercially available degradable polymers [47].

One important advantage in addition to the chemical nature of HYAFF® is the possibility of controlling the rate of polymer resorption and consequently the trend of the tissue response. Indeed, the different esters obtainable from hyaluronan possess controllable degradation properties. In the case of partial esters such as HYAFF® 11p75, there is a rapid spontaneous hydrolysis of the ester bonds occurring in the absence of any enzymatic activity and a consistent amount of the material is released during the first week following implantation. The dissolution of HYAFF® 11p75 involves a temporary macrophage-mediated response [48, 49]. In a rat animal model, the disappearance of the material, even though slower than that of purified HA, was found to be completed in less than a month following both subcutaneous [48] and intramuscular implantation [49]. With a similar trend, phagocytic cells involved in polymer resorption were observed only until the end of the second week of implant presence. The increase in stability of the polymer obtained by the higher esterification percentage of the total ester leads to a slow degradation rate. In some implantation studies using HYAFF® 11 film samples, the total ester appeared to be an inert polymer giving minimal response during the first month following implantation [49]. A macrophage-mediated response as a consequence of polymer degradation was observable after 3 months. During this phase, numerous foamy macrophages were noticeable at the implantation site, internalising the dissolved material. However, such response appeared to be mostly confined to the site where the material had been implanted. Additional in vivo studies, performed at Safepharm Research Laboratories [50] on the toxicity and biodegradation of the material following subcutaneous implantation, indicated that the material had mostly disappeared (under the limit of detection of benzyl alcohol by gas chromatography in the implantation site, approximately corresponding to 1 mg of HYAFF® 11) after about 4 months following implantation in 9 out of 10 animals. Clinical signs, bodyweight, food and water consumption were examined and all the animals were subjected to a gross necropsy examination. No evidence of toxicity was detected during the extensive, one-year study following implantation.

The total ethyl ester HYAFF® 7 was found to behave similarly to HYAFF® 11 as far as the in vivo degradation profile is concerned, even though some partial dissolution was already observable 1 month following intramuscular implantation [49]. However, a more intense reactivity to the HYAFF® 7 polymer was noticeable during the first 2 weeks of implantation in comparison to that encountered with the more inert HYAFF® 11. Analysis of the number of leukocytes at the site of implantation by histomorphometrical techniques quantitatively confirmed such observable differences, indicating a large number of neutrophil granulocytes around the implants.

Furthermore, immunohistological methods showed that the cells responsible for the HYAFF® resorption in the rat model mostly belonged to a subset of monocytes/macrophages positive to ED1 monoclonal antibody staining. A similar finding was observed also in studies in the rat animal model using PLLA implants, where ED1-positive cells were noticed in proximity to the degrading material [49]. On the other hand, mature macrophages and histiocytic cells positive to ED2 monoclonal antibody appeared to be distributed in the connective tissue surrounding the site of implantation.

Composite membranes consisting of HYAFF® 11 reinforced with a mesh of HYAFF® 7 showed similar behaviour to that of their individual components when used in subcutaneous implantation preliminary studies in rat [48].

Until now characterization studies have been conducted only on a limited number of selected derivatives and much remains still to be investigated, exploring derivatives with more hydrophobic or active ester groups. It may be that by varying the substituent groups one can create molecules with slow or fast degradation, locally resorbed or rapidly diffusing and excreted.

10. Hyaluronan esters in drug delivery devices

The possibility of using hyaluronan esters for drug delivery purposes has been considered in a series of experimental in vitro and in vivo models. The release of drug from hyaluronan-based devices can be obtained either by covalently binding active alcohols (e.g. hydrocortisone esters) or incorporating the substance to be delivered within the device. Microspheres and thin films of benzyl esters have been found to be the most suitable physical forms for this type of use.

A number of experiments were undertaken to evaluate the release of hydrocortisone from microspheres made of different esters where the drug was either dispersed or bound to the polymer. While the hydrocortisone dispersed readily by diffusing from the microspheres into a well-stirred solution in about 10 min, the release rate of the covalently bound drug was found to be constant (zero order), in certain derivatives taking more than 100 h, and is thought to be controlled primarily by the ester bond hydrolysis [51]. Other experiences of hydrocortisone release have been described with membranes and microspheres of partial hydrocortisone (25%) and benzyl

(75%) ester of hyaluronan. They appeared to suggest that the mechanism of drug release is independent of the device geometry [52]. Joshi et al. [53] described another possibility of controlling drug release by physically incorporating the drug in a HYAFF® membrane subsequently laminated with a second polymer. Studies have even been conducted treating hydrocortisone esters with porcine liver esterase and bovine testicular hyaluronidase singly and in sequence. The enzymatic treatments did not significantly increase the hydrolysis rate of the material [54].

Preclinical in vivo evaluations in a rabbit animal model demonstrated that methylprednisolone carried by a hyaluronan ester preparation can increase the residence time of the drug in the tear fluid when compared to control suspension [55, 56]. However, the release of the steroid drug in human samples of plasma and synovial fluids was found to present a certain variability, probably due to some esterase activity in physiologic fluid samples from patients with inflammatory arthritis [57].

Studies of macromolecule diffusion and release [58, 59] suggested a possible use of HA esters for peptide controlled release. Promising preliminary in vitro results with peptides were later supported by more consistent in vivo studies considering intranasal delivery of insulin in sheep [60] and vaginal delivery of calcitonin in rats [61, 62]. The use of HYAFF® 11 microspheres loaded with insulin produced large and significant increases in the nasal absorption of this peptide, similar to those which were observed for bioadhesive starch microspheres, when compared to Sephadex microspheres. These absorption-enhancing properties of HYAFF® are related to the bioadhesiveness, appropriate size range of the microspheres and release of the drug from the material, and are apparently not due to mucosal damage, as proved by histological examinations [60].

Overall, the esterification of HA offers a double possibility of drug delivery. Firstly, it provides insoluble materials where the drug can be physically trapped. Secondly, it allows the covalent binding of drugs with a hydroxyl functional group directly to the polysaccharide chain. Furthermore, the mucoadhesiveness of HYAFF® materials opens up the possibility of many alternative methods of delivery via nasal, oral and vaginal mucosa [63].

The great interest in HA as an ideal candidate material for drug delivery has also led to the development of other, more complex methodologies which require the functionalization of the polysaccharide [19, 64] with specific linkers for the covalent attachment of a variety of therapeutic drugs.

11. Hyaluronan-based biomaterials in tissue engineering

The occurrence of many diseases which cannot simply be managed by drug administration has always constituted a severe problem for the clinician. Thus, over the past 80 years other therapies have been developed to overcome this medical gap. Historically, the first successful approach in treating this type of disease was cell transplantation. The infusion of homologous blood cells began the era of biological therapy. Later, great improvements in the surgical and immunological fields, allowed the substitution of whole organs, such as kidney, heart, liver, although the small number of donors together with graft rejection represent the major limitations to this practice. Nowadays, cell culture technology has given scientists powerful tools to harvest, isolate, cultivate and expand almost all kinds of animal and human cells. Thus, one might hypothesize that the new frontier for applied clinical research should be the reconstruction in vitro of tissues and entire organs to be eventually transplanted back into the same donor patient [65]. However, experimental evidence demonstrates that cells growing in flat plastic culture devices undergo certain phenotypical modifications, mainly because they are forced to expand only in a two-dimensional micro-environment, lacking the extracellular matrix (ECM) component. This is a limiting condition for almost all cells, since they normally grow and differentiate in a spatial architecture provided by the presence of a typical ECM, which also regulates many cellular activities. In order to overcome these problems particular medical devices, often referred to as biomaterial scaffolds or matrices, have been designed to accommodate cultured cells. The final aim is to obtain an engineered structure which shares similar properties with the native tissue and, once implanted in vivo, regenerates rather than just restoring the damaged site. Currently, the market offers few classes of natural, semi-natural and synthetic biomedical scaffolds which have been tested for their biocompatibility and biodegradability both in vitro in vivo [66–73]. Amongst these various substrata, hyaluronan appears to be a very promising molecule. In fact, hyaluronan has been shown to play a fundamental role during embryonic development [74–85] and wound healing both in adult and fetal life stages [86–89]. This evidence lead us to postulate that hyaluronic acid and its derivatives may constitute a suitable environment for the growth of cells derived from organ biopsies. In particular, stem elements or adult cells can be stimulated to divide and differentiate in this 'embryo-like' environment [90, 91].

Different authors, recognising the potential of HA in tissue-engineering explored the possibility of incorporating this glycosaminoglycan within collagen [92, 93] or polylactic acid matrices [94].

In our laboratories we have tested various formulations of esterified hyaluronan to assess their biocompatibility and biodegradability [32, 36, 48, 49, 95, 96] and the most suitable devices for cell culture were found to be those made with the total esterified derivative of hyaluronan HYAFF® 11 in the form of a non-woven mesh

(composed of 10–15 μm in diameter fibres) or microperforated membrane (about 20 μm thick membrane with laser-made holes, commercially named Laserskin®).

An advantage of HYAFF® 11-based scaffolds is the good cell adhesiveness even in the absence of any coating or surface conditioning treatment often required by other widely used support matrices such as those made of polyglycolic and polylactic [97].

The scaffolds of total benzyl ester are sufficiently stable in aqueous solution to allow incubation with cells for over 3 weeks. Once wet, the benzyl ester loses part of its mechanical strength, more so than other, completely synthetic materials. However, under in vitro cell culture conditions the material maintains its structural integrity, can easily be handled and does not contract as some collagen-based materials do.

12. Developing artificial skin: skin-equivalent devices, epidermal substitutes and dermal substitutes

The covering of burn surfaces and cutaneous ulcers actually represents the most successful clinical application of tissue engineering. The first therapeutic experiences were performed using split- and full-thickness skin explants from the same patient (autograft), a human donor (allograft) or even an animal (xenograft). At least two decades later, we are now able to reconstruct an organotypic structure which is rather similar to intact skin [98–100]. Moreover, the availability of a tissue bank of cryopreserved cutaneous replacements [101, 102] is another major advantage of epithelial cell culturing which would solve the 'chronic' problem of donor site shortage.

The successful cultivation of human keratinocytes was first established in the mid 1970s and subsequent modifications have resulted in a standardised technique nowadays applied in many laboratories worldwide [103, 104]. Despite optimistic clinical reports [105–107], cultured keratinocyte sheets are prone to some technical restrictions which make it impossible to predict the final outcome of each transplant [98, 108, 109]. Major pitfalls in using cultured epithelial cells begin immediately after the enzymatic detachment of keratinocytes from the culture plate prior to implantation. At this stage, release from the plastic surface causes the keratinocyte sheet to shrink, resulting in a reduced covering area. Moreover, cultured cells form a thin and poorly differentiated epidermal layer which is obviously so fragile it is difficult to handle during the transplantation procedure. To overcome these technical problems, many authors have developed different biomaterials to function as a delivery system for keratinocyte seeding on the wound site [108, 110–112]. These include hydrophilic polyurethane, silicone, polylactic acid, and other natural biomaterials such as fibrin glue.

In vitro studies on hyaluronic-acid-based membranes [113, 114], known by the commercial name of Laserskin®, showed that keratinocytes proliferated on these biomaterials in a similar manner to conventional cultures on plastic dishes. The epithelial cells cultured on the membranes were able to differentiate, and after 15 days of standard culture, the formation of several distinct layers starting from a basal proliferative through to an upper keratinized zone was observed.

It has been previously described that Laserskin® membranes are produced with regular laser-made microperforations. These microperforations, which measure 40 μm and 0.5 mm in diameter, help transpiration when the membranes are placed on the wound bed and also allow cultured cells to migrate and proliferate on the lower and upper surfaces of the membrane (Fig. 17). Keratinocytes within these microperforations were found to express a proliferative basal phenotype. These preliminary in vitro results suggested that HYAFF® 11 membranes are a suitable delivery system for cultured keratinocytes.

Epithelial sheets prepared on Laserskin® have already been applied in an experimental model of cutaneous grafting [115] and encouraging clinical results have already been reported [116, 117]. Recently, it has been demonstrated that, with membranes of HYAFF® 11, also preconfluent keratinocyte cultures give good clinical results in terms of epidermal coverage when grafted onto patients with extensive, full-thickness burn injuries [118]. The option of using membranes with preconfluent cultures in the treatment of burn injuries would enable a more rapid availability of the graft for early coverage. The same possibility is not offered by the traditional keratinocyte sheet grafting technique based on cultures on plastic dishes. Indeed, in this case, before application to the patient, keratinocytes have to become confluent, forming a multilayer structure which can be enzymatically removed from the plastic dish.

The microperforated structure of Laserskin® itself, which allows keratinocytes to expand on both the membrane surfaces, represents an advantage over alternative cell delivery systems. On other polymeric supports, in the absence of adequate porosity, cells can grow confluent only on the upper surface of the biomaterial, where keratinocytes have been seeded. For this reason, during application on the patient, care must be taken to invert the biomaterial so that the side with the cells touches the wound bed.

The cultivation of fibroblasts in ECM-like structures is receiving increased attention not only because of the need to provide a dermal substitute to epithelial sheets (see below), but also because these living constructs may be applied in other pathologies such as ligament/tendon repair [119, 120], vascular prosthesis [121], and dermal augmentation [122, 123]. A common way to culture fibroblasts three dimensionally is to provide the cells

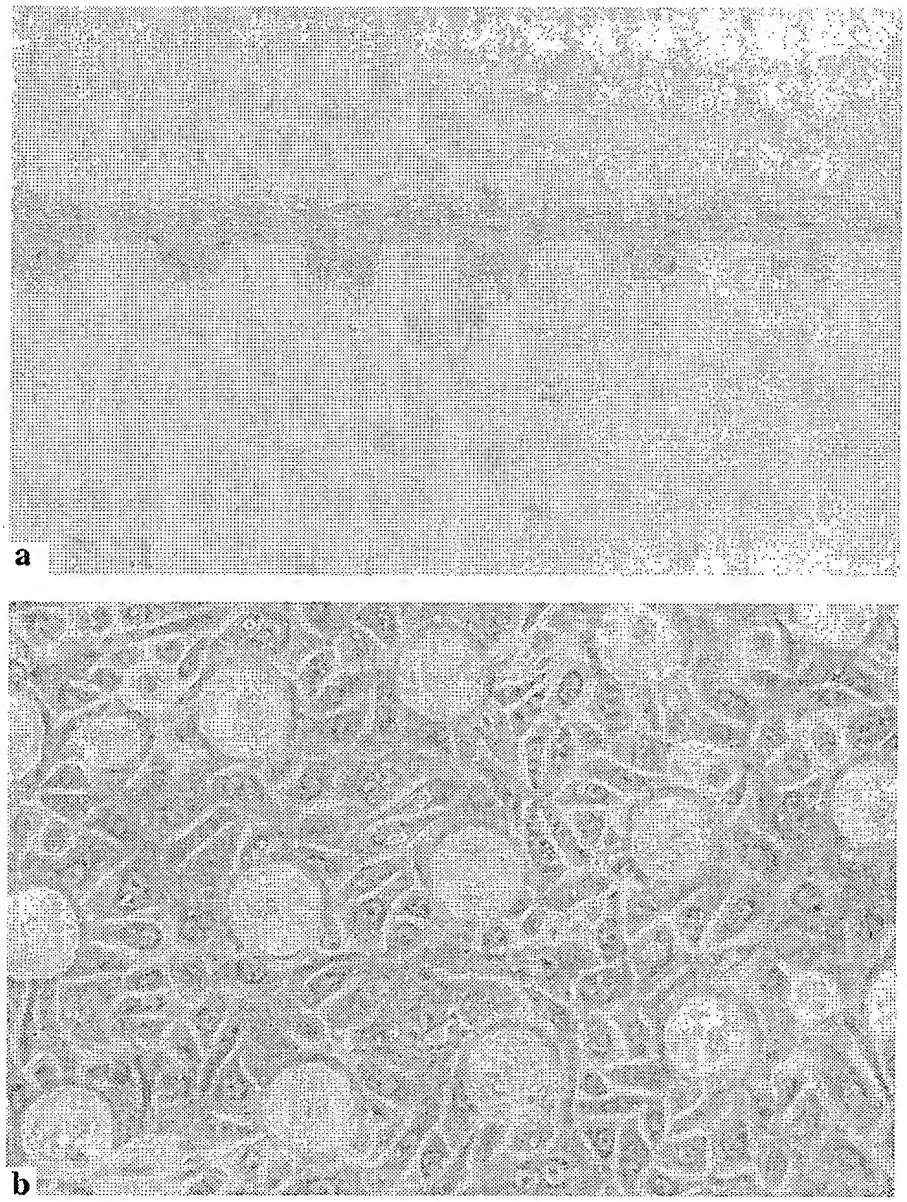

Fig. 17. Micrograph of a HYAFF® 11 membrane seeded with keratinocytes: cross section of the film (a) and phase contrast view from the top (b).

with a collagen-based structure on which to proliferate. However, the major problem with this kind of construct is constituted by the contraction of the matrix [124]. The evidence that covalently-bound or soluble hyaluronic acid significantly reduces collagen contraction in fibroblast cultures [125] is sufficient to consider this glycosaminoglycan as an alternative biomaterial in building dermal substitutes. As mentioned above, fibroblasts have been extensively used to test the cytotoxicity of benzylic derivatives of hyaluronic acid. Given the biocompatibility of HYAFF® non-woven mesh, we isolated human dermal fibroblasts from foreskin biopsies and, after expansion on plastic culture plates, seeded cells at about 1×10^4 cells cm^{-2} on squares (approximately $1.5 \times$ 1.5 cm) of the biomaterial. Cultures were maintained for up to 30 days until the degradation of HYAFF® began, and the growth rate was measured by the MTT cytotoxicity test [126]. As expected, fibroblasts actively proliferated throughout the experimental period, and morphological observations (van Gieson and haematoxylin/eosin stainings) on paraffin-embedded specimens demonstrated that cells migrated through the non-woven mesh and populated both sides of the biomaterials. Interestingly, these produced a distinguishable fibrillar network. Furthermore, immunohistochemical analysis revealed the presence of collagen types I, III, and fibronectin fibres which were evident after only 15 days of culture in the hyaluronan derivative scaffold. These and other

experimental findings clearly showed that HYAFF® non-woven mesh constitutes a suitable environment for the production of a living dermal equivalent. It has been demonstrated that mesenchymal–epithelial interactions regulate several aspects of epithelial development and homeostasis [127]. In fact, several findings support the concept that the dermal component constitutes a permissive and regulatory microenvironment for the growth and differentiation of cultured keratinocyte sheets [128–130]. Composite co-cultures of both fibroblasts and keratinocytes have actually improved the clinical outcome of skin transplants [98, 131–138], and their possible future use for the replacement of current animal models with this organotypic culture as in vitro cytotoxicity assay is strongly encouraged [139]. In our laboratories we have standardised a new fibroblast-keratinocyte co-culture system which may be suitable for application in human full-thickness skin grafting [114, 140]. Cells were obtained from human foreskin specimens, and fibroblasts and keratinocytes were isolated and separately expanded on plastic culture flasks. Fibroblasts were seeded and grown on HYAFF® 11 non-woven mesh as described above. Keratinocytes were seeded at a density of 2×10^4 cells cm^{-2} onto the Laser-Skin® membrane in the presence of a feeder layer of non-proliferating Swiss Balb/c 3T3 mouse fibroblasts. At confluence (10–12 days), pieces of membrane (1.5 × 1.5 cm) were laid down and fixed to the non-woven mesh on which dermal fibroblasts had been grown for 15 days. The fibroblast-keratinocyte composite cultures were laid down carefully on sterile, stainless-steel grids and then cultivated for 15 days at the air–liquid interface. At the end of this period, cultures were harvested and processed for histological analyses. Data from routine histology indicated that several layers of differentiated keratinocytes were formed after 15 days of co-culture. Immunohistochemical investigation of the expression pattern of some ECM molecules showed that in this composite culture several important components of the basement membrane were produced and distributed at the boundary between the epithelial and the underlying dermal layer, such as collagen types IV and VII, and laminin 1 and 5. The formation of a basal lamina with the related anchoring zone is very important to the engraftment rate of a skin equivalent. At the moment, further studies focused on the ultrastructural characterization of the dermal–epidermal junction of this skin equivalent model are being conducted.

13. Artificial cartilage reconstruction by HYAFF® scaffolds

Cartilage is a tissue with only limited self-repairing capabilities. Traumatic and pathological lesions of articular cartilage are very frequent in our aging population and often lead to irreversible degenerative changes causing pain and inhibiting joint movement. Newly developed tissue-engineering techniques appear to offer advantageous alternatives to the current short-lived articular prostheses such as artificial knees, which can represent a valid solution only for relatively old patients and should be considered only in extreme cases.

From a histological point of view, the cartilage tissue consists of a single cell type, the chondrocyte, which can be easily isolated from small biopsies. Chondrocytes can be expanded in culture in a reasonable period of time but tend to lose their characteristic phenotype when maintained in monolayer culture on plastic flasks using conventional procedures. The articular grafting of autologous chondrocytes has already been preclinically and clinically examined in humans by simply injecting cell suspensions within the lesion bed [141–144].

Interesting alternatives to the use of cell suspensions are provided by the possibility of transporting the cells within three-dimensional structures of degradable biomaterials, and of directly grafting them into the chondral lesion. This alternative graft procedure would offer a simplified way of administering the cells. Additionally, the use of three-dimensional scaffolds has been proved to favour the maintenance of the original chondrocyte phenotype under in vitro conditions. The in vitro culture of articular chondrocytes to be used for transplantation has recently been improved by the availability of new, cytocompatible biomaterials [144–148].

Cartilage and synovial tissues have a high HA content. Probably inspired by this observation, purified hyaluronan has been employed as a culture medium supplement and found to favour the maintenance of the chondrogenic potential of chick embryo chondrocytes to be used in animal transplantations [149].

In some preliminary experiments aimed at exploring the possibility of generating artificial cartilage, we used chick embryo chondrocytes harvested from 15-day-old sterna by enzymatic digestion [150, 151]. Primary cell cultures were routinely expanded for 7 days in the presence of sodium ascorbate (50 $\mu g\,ml^{-1}$) and then seeded at 5×10 cell cm^{-2} on HYAFF®11 non-woven mesh. Cells were allowed to grow for up to 30 days and then harvested for histological analyses. The results indicated that avian chondrocytes adhered and proliferated within the biomaterial. They synthesized a characteristic matrix, rich in glycosaminoglycans and collagen and positive to metachromatic toluidine blue stain (Fig. 18), as is usually found in the normal ECM of hyaline cartilage.

Further studies moved to a model more similar to the human situation. A number of papers reported on the possibility of growing bovine chondrocytes on matrices composed of polymer fibres of PLLA and PGA [152–154] obtaining constructs of artificial neocartilage. Following a similar experimental model, bovine chondrocytes from carpal and metacarpal cartilage were

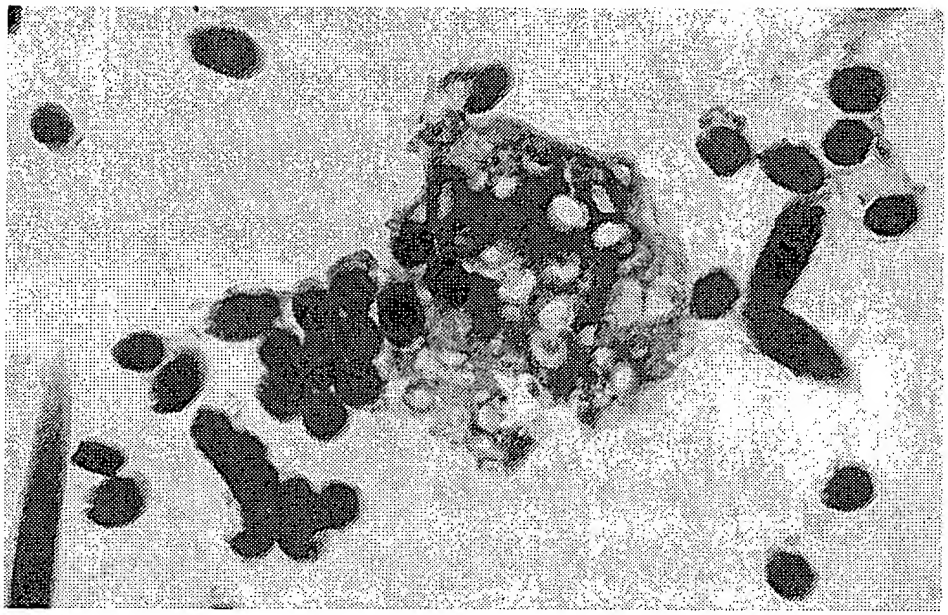

Fig. 18. Micrograph of a non-woven structured material of HYAFF® 11 seeded with chick embryo chondrocytes. The section was stained with toluidine blue stain and shows dark blue stained fibers and a group of chondrocytes which have produced abundant metachromatic matrix.

isolated by enzymatic digestion, expanded and subsequently seeded at various cell concentrations on scaffolds of non-woven structured materials of HYAFF® 11. The construct of HYAFF® 11 was cultured with the cells for periods of up to 7 weeks. The still unpublished results indicate good adhesion of the cells to the fibres of HYAFF® Fig. 19a) even in the absence of surface conditioning treatments often necessary with other polymeric materials. The cells appeared to proliferate, covering the scaffolds homogeneously, and more interestingly to produce a mucopolysaccharide-rich matrix resembling that of the original hyaline tissue (Figs. 19 and 20).

More detailed studies were finally conducted with human cells isolated and cultured using procedures similar to that described above. Cell viability and phenotype differentiation were monitored at different time points during the culture of chondrocytes. The results obtained confirmed good adhesion of the cells on the material. Furthermore, the cells remained viable and able to proliferate within the HYAFF® matrix [155]. The cells appeared to grow over the entire non-woven scaffold and were found again to express their original phenotype, lost during the phase of expansion in monolayer, synthesizing collagen type II.

Observations while culturing the materials with these cells under in vitro conditions indicate that the presence of chondrocytes may influence the stability of the HYAFF® 11 material. It has been noticed that with high cell seeding concentrations the degradation rate of the material and its associated hydration are slightly reduced. Probably, the chondrocytes surrounding the fibres act as a selective membrane limiting the hydration of the polymer. Additionally, the cells, in particular those producing abundant extracellular matrix, secrete proteins which adsorb onto the polymer surface, perhaps with a stabilizing effect on the material. The pH in the interstitial milieu between cells and fibres may be another factor capable of influencing the in vitro stability of HYAFF®. Indeed, as reported by Iannace et al. [31], with time HYAFF® fibres undergo deterioration in neutral and in alkaline (pH = 10.1) solution, but they appear to be more stable in an acidic (pH = 4.01) environment.

14. Mesenchymal stem cell cultures

A new intriguing chapter in the tissue engineering field was opened by the observation that pluripotent cells, referred to as (putative) mesenchymal stem cells, capable of giving rise to several kinds of differentiated elements (myoblasts, chondroblasts, osteoblasts, adipocytes, fibroblasts), are sparsely distributed in many tissues of the body [156–158]. Given this, one would think these embryonic precursors could theoretically regenerate, per se, that part of the adult tissue which has been damaged or lost. Although certain human organs, such as the liver, can effectively regenerate, almost all other tissues tend to form a reparative emergency structure. Moreover, the latter often significantly differ from the native tissue in respect of ECM composition and physiological performance (e.g., fibrocartilage vs normal cartilage). The failure of tissue regeneration in the adult has been pos-

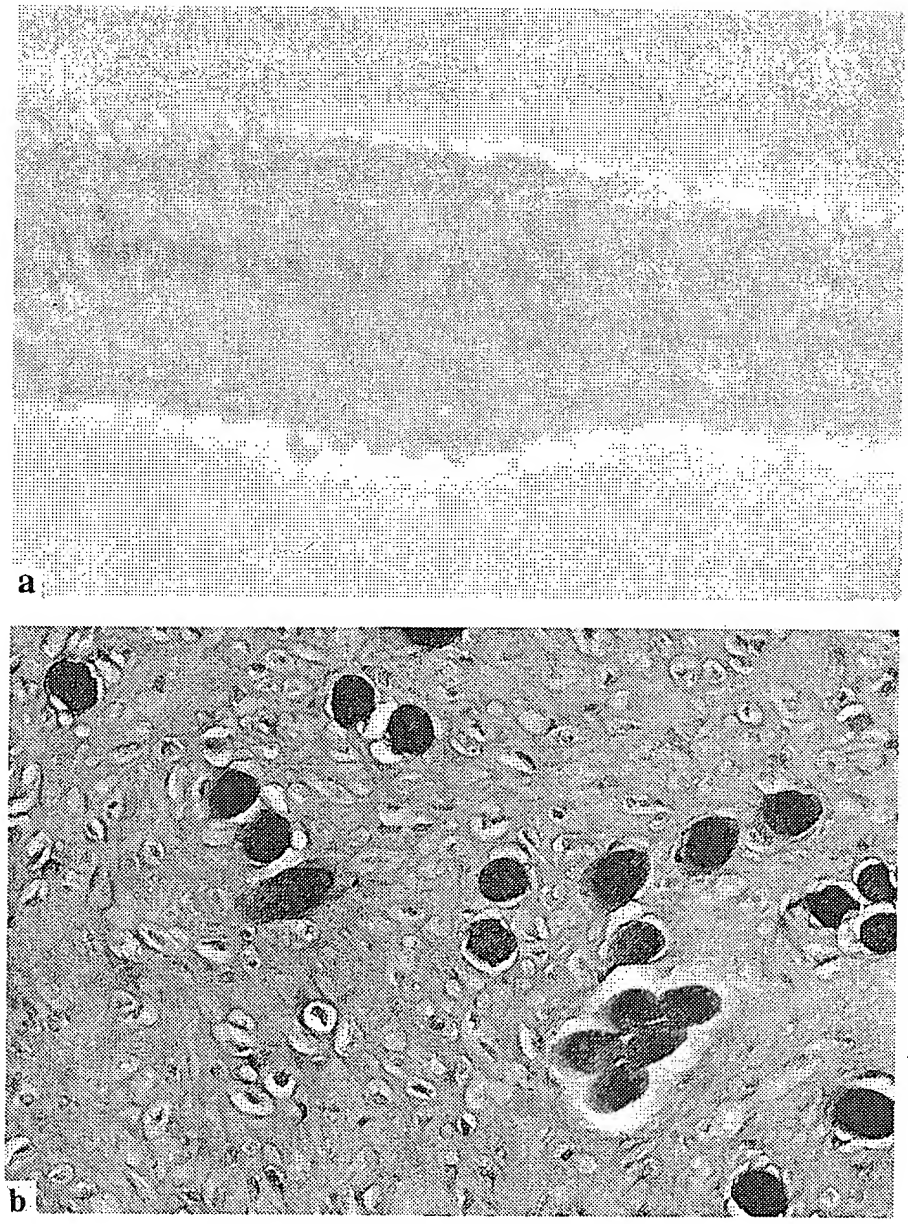

Fig. 19. Bovine chondrocytes cultured on non-woven structured HYAFF® 11: phase-contrast micrograph of chondrocytes adhering onto a single HYAFF® 11 fiber (a), and alcian blue stained cross-section of the material seeded with cells (b). The section shows a few darkly stained fibers. Bovine chondrocytes have grown filling the empty spaces in the three-dimensional structure and appear to be surrounded by alcian blue positive extracellular matrix.

tulated to be due to the lack of sufficient mesenchymal stem cells required to rebuild the damaged structure [159, 160]. If this is true, the potential in using these pluripotent mesenchymal precursors in wound healing is tremendous, given also their large availability from a simple red bone marrow needle biopsy [161]. At this point two possible therapeutic approaches may be followed: mesenchymal stem cells collected and expanded in vitro may either be (1) implanted at high concentrations in damaged sites, postulating that the local microenvironment would force the embryonic cells to differentiate into the proper phenotype; or (2) cultured with appropriate factors to obtain the desired tissue which would subsequently be implanted [162–164]. Actually, the first experimental applications have been performed only on hard connective tissues of animals, such as bone and cartilage [165, 166]. As outlined above, hyaluronic acid constitutes an embryo-like environment in vitro so it would certainly be appropriate for the cultivation of mesenchymal stem cells. Our preliminary data based on cell growth seemed to support this role for HYAFF® 11 biomaterials. Rabbit and human stromal cells were

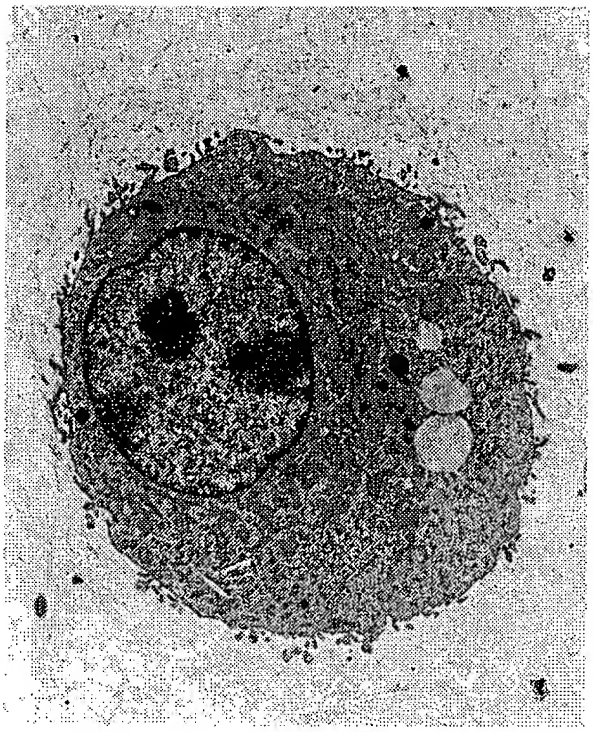

Fig. 20. TEM micrographs of a bovine chondrocyte cultured on the non-woven structured HYAFF® 11. Notice the typical morphology and the progressive deposition of collagen fibrils.

aspirated from red bone marrow (1–1.5 ml). Following the protocol of Lazarus et al. [161], mesenchymal stem cells were expanded with the sole addition of bFGF growth factor in order not to stimulate any particular phenotype expression. Preliminary experiences which need to be substantiated, monitoring the viability of the cells cultured within HYAFF® 11 scaffolds and the induction of their differentiation towards a chondrocyte phenotype, indicated the production of collagen type II. Interesting findings on the suitability of the use of HYAFF® 11 scaffolds with mesenchymal cells have recently been reported by other authors too [167, 168], even though at present it has to be definitively proved whether the inductive effects on progenitor cells are mainly due to the chemical characteristics of HYAFF® 11 or whether they are partly associated with the efficient loading and cytocompatibility properties of the material.

15. HA derivatives for the prevention of bioadhesion and foul-resistent devices

Ongoing preclinical studies, which are now to be published, have achieved successful results by exploiting the poor cell adhesion and degradation characteristics of certain hydrated polymers, in particular of the inner ester ACP, to develop treatments for the prevention of postoperative adhesion formation.

Other applications based on the hydrophilicity and minimal cell adhesion of highly concentrated HA and its derivatives with low percentages of esterification involve the coating of medical devices. Some studies have demonstrated a striking anti-adhesive effect of HYAFF® 11p50 coatings, obtained through a process of surface activation by plasma treatment (glow discharge) and a coupling reaction, not only on mammalian cells but also on bacterial strains such as *Staphylococcus epidermis* [169]. This would suggest that in addition to the above-mentioned applications, HA partial esters may offer a valid solution where undesired bioadhesive phenomena have to be prevented.

Among the ideal candidate devices where coating with HYAFF® appears to be beneficial are trans-mucosal devices, such as titanium abutments, and intraocular lenses.

16. General discussion

This review deals with the biocompatibility of some hyaluronan derivatives. It is clear that these derivatives have considerable potential as biomaterials. They may be prepared with varying degrees of stability, ranging from readily water-soluble to solid polymers with in vivo lifetimes measured in months. In order to determine the value of these polymers in medical applications, it is necessary to establish as much as possible about their biocompatibility properties. Biocompatibility was defined at the Consensus Conference of the European Society for Biomaterials [170] as 'the ability of a material to perform with an appropriate host response in a specific application'. This implies that, at the very least, the material should have no adverse effect on the host but, equally importantly, that it should initiate or promote those events in the tissue which are most beneficial for its function in the application for which it is intended. These events are concerned with either the proteins that come into contact with the material or the cells which it is able to influence, or more usually both of these factors. We may therefore consider biocompatibility in terms of the potential not to do harm, i.e. induce toxicity, and the potential to interact with tissue components to produce the most favourable response. In the latter case, this potential for interaction can relate to the cellular components of tissue, to the molecules of the extracellular matrix and the tissue itself.

With respect to cytotoxicity, there is no doubt that the HA derivatives, and in particular HYAFF® 11, appear to be cytocompatible polymers, as proved not only by the conventional cytotoxicity screening procedures but also by extensive experimentation with many different cell types. Only in the case of the partial benzyl ester HYAFF® 11p75, which undergoes rapid degradation, has a slight reduction in cell activity been observed with

14-day extracts. As regards the interactions between solid materials and cells, a number of factors may be involved. The studies reported here have addressed the phenomena of cellular adhesion to polymer surfaces, their migration or movement over the surfaces and activation during or after contact or otherwise under the influence of the material.

HA partial esters with a low percentage of esterification such as HYAFF® 11p75, but also ACP, or hydrated esters such as HYAFF® 7 were generally unattractive to fibroblasts which showed little affinity for their surfaces. HYAFF® 11 was the only material screened among the derivatives explored onto which the cells were able to adhere and spread.

HYAFF® 11 is very much the least water-absorbing material of this group and it has been shown elsewhere that high levels of water uptake may be in some way associated with poor cell adhesion [35]. In a report on cell attachment to various natural and modified collagen preparations, the incorporation of HA was shown to improve the adhesion properties of collagen based sponges [67]. However, HA concentrations exceeding 5% were reported to have the opposite effect, inhibiting fibroblast adhesion. Little is known about this inhibitory effect. One possibility is that there is a specific physical interaction between the cell glycocalix and the strongly hydrated and negatively charged HA molecules. It is possible that polymers such as ACP, the partly esterified HYAFF® 11p75 and the total ester HYAFF® 7 present only limited modification with respect to the HA structure and its hydration properties. When highly concentrated in the form of a film or sponge they might not then allow the cells to adhere.

The structure of HYAFF® 11 is characterised by the presence of numerous hydrophobic benzyl groups along the polymeric chain. These groups are probably organised in hydrophobic patches and probably give the molecule a more rigid and less mobile conformation, reducing its ability to interact freely with water.

A series of studies in which leukocytes were placed in contact with HYAFF® 11p75, HYAFF® 11 and HYAFF® 7 suggest that the total benzyl ester HYAFF® 11 is an inert material which does not significantly alter the neutrophil morphology or locomotion, or the level of macrophage metabolic activation.

The experiments show that neither solutions of high molecular mass HA nor suspensions of HYAFF® 11p75 and ACP are capable of causing neutrophil polarisation. These results are consistent with those of other studies showing that high molecular weight HA has no effect on neutrophil activation. Moreover, the data indicate that the partially esterified HA derivative does not activate neutrophils and is not recognised as active low molecular mass HA fragments, as may be the case with partial esters. Such materials, therefore, should not by themselves constitute an inflammatory stimulus for neutrophil granulocytes when they are placed in the physiological environment.

On the other hand, HYAFF® 7 was the only material able to cause neutrophil chemokinesis with effects similar to those reported for the positive control in which the cells were stimulated with fmlp-peptide.

The experiments concerning complement activation confirmed the inert nature of HYAFF® 11 with respect to all the other derivatives examined. The moderate production of Bb, iC3b and SC5b-9 by HYAFF® 11p75 and HYAFF® 7 gave consistent results indicating moderate activation. From the data, it appears that the enzymatic cascade was not triggered at the level of the classical pathway, but rather that the alternative pathway may be the preferential site of activation. This is consistent with complement activation associated with the surfaces of biomaterials and medical devices in general, where it is believed that the alternative pathway dominates [171, 172].

In the case of interaction with fibrinolysis too, complete esterification with benzyl alcohol appeared to reduce the effects of interaction with fibrinogen observable in the case of native HA.

Although in vitro experiments are extremely valuable in providing data to assist in the understanding of biomaterial interaction with proteins, cells and other components of the tissue, they cannot reveal how the materials will interact with all of these components when they are assembled in the form of the tissue itself. In order to get a better idea of material–tissue interactions, it is necessary to conduct experiments in animals. A number of procedures were performed in which the totality of the in vivo interactions were considered in certain animal models.

With respect to degradation kinetics, substantial differences were observed between the two total esters and HYAFF® 11p75. This may be accounted for by the differing hydration properties of these polymers favouring the hydrolysis of ester bonds, and is consistent with degradation profiles in artificial plasma. Furthermore, according to experiments on the in vitro degradation of HYAFF® products by hyaluronidase, polymers with a lower degree of esterification are also slightly susceptible to depolymerization by this enzyme [95]. In the rat intramuscular and subcutaneous model the presence of implanted HYAFF® 11p75 and the associated tissue response appeared to have completely subsided after about a month.

Both of the total esters had a slower degradation rate. In the case of HYAFF® 11, only slight dissolution of films was observed after a month of implantation, while HYAFF® 7 showed clear evidence of degradation at this time. Three months after implantation, large numbers of macrophages were observed at the site of implantation as would be expected with a degrading material. However, the tissue response appeared to be confined to the site of implantation. Furthermore, long term implantation

studies showed no local or systemic effects of any importance associated with HYAFF® 11 material [50].

The in vitro data may help to interpret these findings. HYAFF® 11 appears to be a very inert material for up to one month. Its low reactivity increases as soon as degradation starts to take place, probably because partial esters are formed as hydrolysis of the ester bonds occurs. This degradation and the release of degradation products may activate the complement cascade, with opsonisation and subsequent recruitment of macrophages. More extensive studies with other degradable polymers could probably ascertain whether this mechanism, by which phagocytes may be recruited by leachable degradation molecules, is normally involved in the resorption of most common polymers.

The particular chemistry of hyaluronan esters compounds and their kinetics of degradation offer a possibility for drug delivery of peptides and active alcohols either dispersed in the medical device or covalently bound. However, it is in tissue engineering that these HA derivatives may find their most promising clinical applications. Because of their particular cytocompatibility, HYAFF® derivatives and especially HYAFF® 11 have been found to be suitable substrates to grow a variety of cell types including fibroblasts, keratinocytes, chondrocytes, endothelial and mesenchymal cells.

Membranes and non-woven structured materials made of HYAFF® 11 allow good cell adhesion and viability. Even more important for those cells which under normal culture tend to lose their differentiated phenotype, the HYAFF® 11 three-dimensional scaffolds appear to favour the maintenance and re-expression of their original assets [155].

ACP and other hydrated partial esters with low cell adhesiveness have recently been considered for the prevention of surgical adhesions and as foul-resistant coatings for medical devices, thus extending the potential of HA esters in medical applications.

A variety of degradable biomaterials have now become available for clinical use. Among them there are some synthetic materials such as polyorthoester, polylactic and polyglycolic acid, polyhydroxybutyrate and polyhydroxyvalerate, polycaprolactone, polydioxanone, polyamino acids and polygalactose, some derivatised natural polymers such as hylans and extracellular matrix analogs consisting of cross-linked collagen and glycosamino glycans, and a number of unmodified natural materials such as fibrin, collagen and hyaluronan.

With respect to other degradable materials, HA esters possess both the processability of completely synthetic polymers and the advantages related to the use of a highly purified natural polysaccharide. Their production does not require cross-linking reagents as in the case of other natural material derivatives, but a simple coupling reaction which can be stoichiometrically controlled and involves the formation of hydrolysable ester bonds.

The vast majority of the cross-linked HA derivatives mentioned previously are represented by very hydrated materials which are not cell-adhesive and, for this reason, were initially considered as resorbable materials for the prevention of surgical adhesion. A few attempts to produce adhesive HA-based matrices have been made but involved the attachment of Arg–Gly–Asp (RGD) peptides to cross-linked HA materials through bridge molecules [173]. Alternatively, the esterification of HA can provide materials which, depending on the ester group introduced and the extent of esterification, can also exhibit limited hydration and sometimes promote cell adhesion, as in the case of HYAFF® 11.

The biocompatibility of resorbable materials requires fine tuning of the degradation rate and of the loss of mechanical strength in order to enable tissue repair and regeneration to progress simultaneously. This obviously depends very much on the specific type of clinical application. Hyaluronan esters offer a range of polymers with different controllable susceptibility to degradation. However, like the majority of resorbable materials they have rather limited mechanical strength when they are wet. Only a limited number of degradable synthetic polymers such as self-reinforced composites based on glycolic acid exhibit adequate physical properties to be considered for hard-tissue implants which must be resistant to considerable stress. Neverthless, too rapid loss of stiffness and sometimes non-infectious inflammatory response have been reported for these biomaterials [174]. In the orthopaedic field especially there is a real interest in biocompatible materials with appropriate strength and degradation profiles for bone and tendon repair. Synthetic polyamino acids and pseudo-polyamino acids based on amino acids coupled through nonamide bonds appear to be very promising for their particular mechanical strength and their chemical similarity to natural molecules.

Ongoing studies aimed to develop biomaterials with improved mechanico-physical properties are also investigating the production of hyaluronan esters with more hydrophobic substituent groups and increased stability.

17. Conclusions

All the studies reported here show that the polymeric derivatives of hyaluronan constitute a variable and versatile group of biodegradable materials. They can be produced with varying chemical structures and morphology, such that the rate of degradation, the degree of hydration, the cellular responses and the overall tissue responses can be manipulated.

The materials are intrinsically safe to use since little or no evidence of cytotoxicity has been found. On the other hand, a range of positive interactions with tissue components, primarily associated with cell adhesion and

migration has emerged. The nature of these interactions appears to vary with the degree of esterification. This does not necessarily imply that there are any significant differences in surface characteristics, indeed, there appears to be little change in surface energy and protein adsorption behaviour. The effects are more likely to be associated with differences in hydration and degradation rates. In the former case, the degree of hydration would appear to have a significant influence on the ability of cells to adhere and migrate over the surface. In the latter case, the rate of degradation, which is controlled by the ease of access of either water or active species to the degradable bonds, is likely to influence cell activation because of the inflammatory stimulus that it provides. These factors are all mutually interactive and interdependent so that there is no obvious or clear association between any one material parameter and biological response.

An interesting point arises in the general consideration of biocompatibility. The first concerns the comparison between the activity and behaviour of different cells on similar material substrates. It is often stated that protein and cell behaviour at material surfaces is governed by certain surface characteristics, such as hydrophilicity, surface charge and surface energy. It has long been known that the varied characteristics of proteins mean that there are no clear, unequivocal relationships that universally govern, say, protein adsorption parameters and surface features. However, cellular behaviour is normally considered to be a little more consistent. It is clear from these experiments that, with the chemical structure of these hyaluronic acid derivatives and their physical characteristics, cell adhesion may be quite different with different cells.

Very little has been explored within the new class of polymers of HA derivatives and much remains to be investigated about the properties of new esters with more hydrophobic substituents or with a combination of different substituent groups to form mixed esters. Further studies are expected to widen the range of HA-based materials suitable for medical application.

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